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THE EVOLUTION OF ADAPTIVE IMMUNITY IN VERTEBRATES

By

F M BURNET

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Any attempt to trace the evolution of a functional system in mammals must necessarily be speculative—it is also likely to have an out dated flavour as of something more suited to the intellectual climate of the 1890 s than of today Yet it is still arguable that it is impossible to understand any aspect of living function unless it can be seen against an evolutionary background

I have a rather special personal interest in the evolution of the immune processes which are the subject matter of conventional mammalian immunology In 1957 I sketched a clonal selection theory of immunity (1) and since then I have spent most of my work and leisure in keeping the general approach embodied in that theory up to date This has meant a continuous process of modification so that in many ways the account differs from that of 1957 but yet it is still basically a selective theory My impression of current immunological thought as expressed for instance at the Cold Spring Harbor Symposium on Antibodies in 1967 was that instructive theories were no longer admissible but that a thorough going clonal selection theory was very distasteful to scientists nearly all of whom were biochemists by training This may have been mainly because of the difficulty of subjecting such a theory to a crucial experimental test

My own attitude has become progressively to believe that if one looks at the over all picture of immunity there is no alternative to a clonal selection approach in the broad sense Accepting this my main interest has been to look at the implications of the theory for the understanding of the less experimentally accessible aspects of immunology notably immunopathology and the evolution of adaptive immunity Here I am concerned to discuss how adaptive immunity a characteristic only of vertebrates may have arisen in the early stages of vertebrate evolution

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Substance of a lecture given on May 29 1968 to the Danish Society of Pathology at the State Serum Institute Copenhagen

Current Immunological Theory

The essence of an up dated selection theory of immunity is that in the line of cells ancestral to immunocytes there is generated by some type of genetic process a diversity of immune pattern This is followed by phenotypic restriction so that a newly differentiated immunocyte can produce only one pattern of immunoglobulin with the same pattern persisting in any descendant clone Such cells carrying immunoglobulin receptors recognize or react with any antigenic determinants which have enough steric complementarity to the immunoglobulin combining site to allow union which can stimulate the cell to significant function

A more recent development which is of vital importance to any evolutionary discussion has been the division of adaptive immune processes into two rather sharply differentiated systems Without attempting more than a dogmatic statement of the position these are

1 *The thymus dependent system of immunocytes responsible for the phenomena of homograft immunity delayed hypersensitivity and immunological surveillance These cells produce no more than trace amounts of immunoglobulin*
2 *The GALT dependent system of immunocytes in which GALT (gut associated lymphoid tissue) represents the still incompletely identified mammalian equivalent of the avian Bursa of Fabricius* These are responsible for the appearance of plasma cells with their specialized mechanism for producing large amounts of immunoglobulin (antibody)

Perhaps the clearest way of appreciating the difference between the two systems is to compare the immunological capacities of the child with congenital agammaglobulinaemia with those of a normal child In affected children there is no appearance of plasma cells in an antigenically stimulated lymph node there are only traces of circulating immunoglobulin and no demonstrable antibody production The chief weakness of these children at the clinical level is their inability to handle bacterial infections of the respiratory tract The course of an attack of measles however shows no significant difference from the picture in normal children apart from failure to produce antibody Other aspects in which such children behave normally are in rejecting homografts becoming sensitized to simple chemicals such as dinitrochlorobenzene and showing normal thymus histology and normal numbers of circulating lymphocytes These characteristics are all in accord with Good s interpretation of the condition as resulting from the genetic absence of some substance or function necessary for the development of the GALT dependent system (12)

As will emerge in later discussion there can be no doubt that the thymus dependent system is the more primitive As one indication of

this there appears to be no genetic abnormality which can be interpreted as an uncomplicated absence of the thymus dependent system

To complete the broad outline of current theory mention must be made of the phenomena of immunological tolerance If immune pattern is developed by a random process of diversification it is necessary consequence that a proportion perhaps a large proportion of combining site patterns will be reactive with potential antigenic determinants of the body s own components A mechanism must exist by which such forbidden patterns must be eliminated The obvious suggestion (2) is that when newly differentiated immunocytes meet an antigenic determinant with which they can react they are destroyed The alternatives that they are rendered functionally inert or switched to another pattern seem highly unlikely but would be operationally equivalent to destruction This capacity to eliminate self reactive clones must be common to both systems An interesting example from human pathology in which acquired tolerance to a given antigen is developed by the T D system but not by the GALT D system is to be seen in progressive measles virus infection of the brain in the rare condition subacute sclerosing pan encephalitis (5)

Defence Processes in Invertebrates

Despite the relative inactivity of research on immune responses to infection nowaday the concepts of classical immunology were all developed in relation to infectious disease An attack of mild smallpox protected against subsequent infection with smallpox but not against measles and *vice versa* This is the essence of adaptive immunity a specific response to one type of antigen only It is characteristic of vertebrates only

This immediately raises a fundamental question How have invertebrates managed to survive without any form of adaptive immunity? There has been extensive study of the responses of invertebrates to bacterial infection to metazoan parasites and less extensively to transplantation of tissue (*e g Salt* (14 15) *Tripp* (18)) One can probably summarize the situation in invertebrates by saying that they have a fully adequate capacity to prevent invasion of their tissues by potentially pathogenic micro organisms to deal with the early stages of established infection and within limits to repair traumatic or infective damage It is clear that there is a certain capacity to recognize foreignness probably inherent in the mobile cells—haemocytes—of the body cavity Foreign material inserted into the cavity is dealt with by phagocytosis or encapsulation but there is no evidence that experience in dealing with one type of bacterium results in any improved capacity to respond specifically to that bacterium on a subsequent occasion There are often antibody like actions of invertebrate body fluids in for example agglutinating mammalian red cells but nothing at all equivalent to antibody has ever been demonstrated

The essential features of mammalian immune systems are

1. A thymus and probably another primary lymphoid system
2. Circulating lymphocytes
3. Immunoglobulins and the plasma cells which synthesize and liberate them
4. Capacity to produce antibody on antigenic stimulation
5. Capacity to reject tissue homografts

The possession of one or more of these criteria indicates an adaptive immune system achieved or in the making. None have been established for any invertebrate.

It is possible to express the difference between invertebrate and higher vertebrate in terms of cellular reactions provided we make the reasonable assumption that the haemocytes of invertebrate body cavities are homologous with and in a certain sense ancestral to the complex of phagocytic cells and immunocytes found in the vertebrates. In the vertebrate the capacity to recognize foreignness, i.e., difference from self structure is much more refined than in invertebrates. Only in vertebrates do we find an ability to reject skin grafts from other individuals of the same species. Further, there is the essential difference that in a bird or mammal, stimulation of a specifically patterned immunocyte under appropriate conditions will lead to the production of a descendant clone of the same immune pattern; no such proliferation has been seen in an invertebrate.

The Immune Responses of the Lower Vertebrates

Most immunological experiments have made use of the common laboratory mammals but work on the domestic fowl and a few other bird species shows essentially similar capacities. Cold blooded vertebrates have been looked at by fewer investigators but it is clear that amphibians and reptiles, cartilaginous and bony fish can all produce antibody and reject homografts, have a thymus and circulating lymphocytes and have immunoglobulins with many chemical similarities to mammalian ones (16).

Next below the fishes in the evolutionary scale are the Cyclostomes, eel shaped marine or fresh water animals with a notochord but no true vertebrae, lacking jaws and now represented by lampreys and hagfish.

There are numerous species of lamprey but largely as a result of its economic importance in the Great Lakes of North America practically all immunological studies have been made on the marine lamprey *Petromyzon marinus*. The larval forms of all lampreys are filter feeders living like many small invertebrates on microscopic organisms in water or mud of fresh water streams. After metamorphosis the adults of *Petromyzon* normally move down stream to the sea but they can also complete their life cycle in fresh water lakes. In either environ

ment they are parasitic on fish They attack by their sucker mouth and drill a hole into the tissues with the rasp like tongue They feed primarily on blood but also liberate a proteolytic secretion which produces local tissue damage

Two genera of hagfish have been studied *Eptatretus* and *Myxine* (7) These are also parasites on fish particularly injured or immobilized ones as well as scavengers Like lampreys they penetrate the body but once inside devour everything but bone and skin

Investigation of lampreys and hagfish from the immunological angle has been largely limited to the work of *Good* and his colleagues (*Papermaster et al* (11 10) *Finstad & Good* (6 7)) but the results reported seem to be definitive in showing that the lamprey possesses a primitive and relatively inefficient immune system The possibly more primitive hagfish gave no positive findings One gathers however that it is a very difficult animal to maintain in the laboratory and there is a possibility that undermore natural conditions some positive responses might have been obtained In the lamprey *Finstad & Good* (6) found that antibody was produced to a Brucella vaccine but not to 9 other standard antigens including phage T2 In the epithelium of the pharyngeal gutter there are small groups of lymphoid cells which may represent a thymus and there are circulating cells resembling large and medium lymphocytes There was a clear differentiation between skin autografts and homografts All */ autografts were retained indefinitely the same number of homograft experiments all showed rejection in 3 to 6 weeks Sensitization to tuberculin by injection of Freunds complete adjuvant was also demonstrated It was notable that in these experiments a high degree of local sensitization often followed by ulceration developed

This series of experiments shows that in the lamprey the reactions which are the responsibility of thymus dependent immunocytes in mammals are much more clearly demonstrable than antibody production This is also seen in fish although antibody production is considerably more versatile than in lampreys Nothing is on record about immune responses in more primitive Chordata

The only evidence available therefore points strongly to the conclusion that the adaptive immune system evolved first in primitive cyclostomes that its essential characteristics took form then and have been maintained in all subsequent vertebrate forms This immediately poses the question What evolutionary need was responsible for this far reaching functional change— the appearance of an adaptive immune system initially with qualities analogous to the thymus dependent system in mammals?

The Evolutionary Need

If there is no weakness in the ability of invertebrates and primitive chordates like Amphioxus to protect themselves from micro organismal invasion why was it necessary to evolve a new adaptive system for the

vertebrates? A closely related question which probably provides the clue to the answer is Why do vertebrates not only have the capacity to recognize foreign tissue from another individual of the same species but also produce a wide range of antigens within the species which allow this recognition to take place? The first suggestion that immunity was not wholly concerned at the evolutionary level with defence against infection was due to Thomas (17). He raised the question whether the remarkable ability to differentiate between fine chemical differences might have been related to either of two other circumstances of great evolutionary significance the position of the foetus as a virtual parasite of the mother's tissues and the prevalence in mammals and birds of malignant disease also equivalent to a parasitic cell population living at the expense of the host. The maternal foetal relationship occurs aberrantly in a few species of fish but only became standard in the higher mammals. It could not have been concerned in the early development of the immune mechanism. For several years I was deeply impressed with the potential importance of cancer as an evolutionary agent. A cancer arising in a pure line mouse can be transferred by inoculation to any other mouse of the same genetic quality but more easily to a newborn than an older animal. If there were no histocompatibility differences between human beings cancer arising in old people and involving primarily or secondarily the surface tissues would be infectious for any mildly traumatized areas in children. Such a process if it could arise would rapidly produce an evolutionarily intolerable situation. There are other reasons too why adaptive immunity might have as its most important function the ability to recognize and destroy mutant somatic cells arising in the body. This surveillance function has been discussed elsewhere (3). Looked at purely from the human (mammalian) angle a very strong case can be made for believing that the most important function of adaptive immunity is as an incomplete protection to the species against any form of malignancy taking on an epidemic form.

Adaptive immunity however is not a mammalian discovery. It must have emerged first amongst the cyclostomes or their ancestral forms of Ordovician and Silurian times. Cancer only becomes potentially important in large animals living for long periods and having frequent bodily contact of a sort which could allow transport of viable cancer cells from one to the other. Free swimming marine or fresh water animals producing large numbers of offspring mostly destroyed by predators do not fit into the picture. While worrying over this discrepancy I began to think about the uniquely parasitic characteristics of both classes of present day cyclostomes. Here perhaps was a clue.

Cyclostome Evolution and Adaptive Immunity

Until the mid 1930's there was a flourishing and economically important fishing industry in Lake Superior and the other Great Lakes of

North America based mainly on the lake trout (*Savelinus*). Within ten years the trout were virtually exterminated and the industry ruined by the entry of the sea lamprey (*Petromyzo* on) into the lakes. There are several points of interest about this occurrence. All lampreys spend their larval life in fresh water streams and though most adults after metamorphosis migrate to the sea they are equally capable of spending their adult parasitic period in fresh water. Sea lampreys were present in the lowermost of the Great Lakes, Lake Ontario, presumably since its formation but they had been unable to pass upwards beyond Niagara Falls. Apparently the enlargement of the Welland Ship canal which was completed in 1932 provided the opportunity for lampreys to reach the other lakes and to find suitable places for spawning and larval growth in their tributary streams. Destruction of fish in the newly invaded area was virtually complete but it is significant that trout have always been present in Lake Ontario and though many show scars where lampreys have been attached a *modus vivendi* between host and parasite has been established (8).

The fact that all lampreys and hagfishes are parasitic in the adult form—the few non parasitic lampreys do not feed at all during their adult phase—suggests that the habit has been common to the group since the early days of its evolution. If a similar form of blood sucking parasitism arose in the Silurian the only potential hosts with a vertebrate type of blood circulation would have been other cyclostomes. It is easy to conceive of a form like the existent ammocoete larva of the lamprey living on micro-organisms finding an alternative way of living on red cells from larger forms of the same or related species and evolving various forms of parasitism. If at this time the tissue components of host and parasite were so similar that there was no effective way by which the host individual could mount a defence against the parasite an intolerable situation would eventually arise by which the extinction of the host species would automatically also eliminate the parasite. A situation where a group of organisms has become divided into (a) parasites capable of entering the tissues of (b) the host form which was unable to react against the invasion would call for urgent evolutionary developments. We are assuming that the parasite having essentially the same structure as the host it is invading would be in very much the same situation as a cancer cell.

Viewed with the hindsight of what has emerged in vertebrate evolution there are two possible ways of escaping from the dilemma.

1. The host forms might develop an impermeable body coating which would prevent the parasite from entering the tissues.
2. A process by which the parasite could be recognized as foreign might develop.

4. It is of interest that the first vertebrate fossils from the Silurian are of heavily armoured cyclostomes the Ostracoderms in which bony

plates have developed over most of the skin. There are other possible reasons for these plates than for protection against other cyclostomes of parasitic habit and for obvious reasons only armoured forms will be observable in the fossil record.

While this paper was being written an interestingly relevant annotation appeared in *Nature* in regard to what is considered by palaeontologists to be a cyclostome ancestral to both lampreys and hagfishes. This is the middle Silurian form *Jamoytius*, an elongate vertebrate well enough preserved to show a round suctorial mouth with supporting cartilage and a simple branchial basket. It was about 20 cm in length and of an elongate lamprey-like shape but differed from all living cyclostomes in having a single row of scales on each side. It lived in a marine environment and is regarded as an ideal ancestor for the modern cyclostomes (13).

The point of major interest in the present connection is, however, the finding that associated with *Jamoytius* are large carbonaceous sheets of the problematical organism *Dictyocaris* and Ritchie notes that these are often perforated by numerous circular holes 3–5 mm across which could have been caused by *Jamoytius*.

Here then is a clear suggestion that the present suctorial parasitic habit of lampreys and hagfishes goes back to the Silurian, thereby rather strongly supporting the hypothesis that I have presented above.

B. The evolution of a means of recognizing as foreign a closely related type of organism and reacting against it in much the same way as against a grossly different type of parasite would probably be a long and complex process. It would demand that the host species should make itself different from the parasite by a process which the parasite species could not easily neutralize by making the same change. Again making use of hindsight the most promising direction within genetic possibility would be to allow an increased flexibility in the pattern of cell surface components capable of being recognized. A random series of changes in what we now speak of as histocompatibility antigens occurring rather frequently could not be matched by another line or species. Equally important would be the development in the mobile defence cells of complementary patterns capable of recognizing antigen patterns different from self.

It would be unwise to spin a web of speculation about how the process by which immunocytes develop their diversity of patterns may have evolved. That may become a legitimate exercise when we understand the nature of the process as it exists in mammals. I cannot resist, however, raising the possibility that more or less simultaneously there may have developed a greater flexibility or mutability of the genome which could be seized on by evolution (a) at the germinal genetic level to allow a multiplicity of random histocompatibility antigens to arise within a species, (b) at the somatic genetic level to allow also by a random process the development of diversified immune patterns in

immunocytes and (c) perhaps to apply the same principle of diversification and clonal selection to the cellular differentiation of some other organs or cell types (9) It could be equally conceivable that an unwanted manifestation of the same flexibility in the genome has been the emergence of malignant disease

Immunological Surveillance

The hypothesis then becomes that adaptive immunity may have evolved as a response to the development of parasitism by early cyclostomes on hosts of similar character perhaps even on larger individuals of the same species It should be emphasized again that such a situation is closely analogous to that of a focus of neoplastic cells acting as a virtual parasite on the animal in which it has arisen The need in both is the development of a refined mechanism for recognizing foreignness and mounting some form of reaction to destroy or minimize the effect of the foreign cells or organism

The general argument and even the detailed discussion used previously to develop the hypothesis that adaptive immunity arose as a means of minimizing the dangers of malignant disease (17 3 4) would therefore be applicable to the present hypothesis If it should emerge that the same loosening of genetic control that was needed for the development of an adaptive immune system was also responsible for the appearance of cancer as a disease of vertebrates it would be in line with evolutionary principles that the new mechanism of adaptive immunity should be further refined for use against the new danger In a number of papers I have developed the concept of immunological surveillance as an important aspect of the natural history of human cancer It is probably correct to say that one of the major aspects of experimental and clinical research on neoplasia is now concerned with antigenic differences of cancer cells from those of the host and with the possible application of those differences to find ways by which an immunological approach to cancer therapy might be developed In the long run it may prove to be more important to concentrate on possibilities of reinforcing the normal process of surveillance as a preventive of malignant disease than to struggle with the obdurate problems of immunotherapy

CONCLUSION

This paper is essentially an attempt to re emphasize the importance of the function of immunological surveillance as a process without which malignant disease in man would probably be much more frequent and involve younger age groups An obvious weakness of this concept of surveillance was the absence of any credible mechanism by which adaptive immunity capable of surveillance function could have evolved in early marine vertebrates presumably of cyclostome type

It may never be possible to obtain decisive evidence in favour of the hypothesis of parasitism of cyclostomes by closely related smaller forms The idea however brings into reasonable relationship a range of facts which would otherwise seem to have no mutual relevance viz

1. The lamprey as the most primitive animal to show adaptive immunity
2. The much greater importance of thymus dependent as compared to GALT dependent immune function in all the lower vertebrate forms
3. The parasitic character of most existing cyclostomes and almost certainly of at least one Silurian form
4. The armoured skin of many Silurian cyclostomes (Ostracoderms)
5. The demonstration of the evolutionary potentiality of cyclostome parasitism in the Great Lakes 1935 55

The additional suggestion that the evolutionary solution adopted which required the development of multiple histocompatibility antigens as well as of mobile cells carrying immune pattern took the form of an increased flexibility of genomic control is equally speculative It could however have heuristic value for the understanding of cellular differentiation and of the basis of malignant change in cells

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ANEURYSM OF THE AORTIC SINUS OF VALSALVA

A Case of Rupture and Myocardial Infarction

By

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Received 29 x 68

Aneurysms of the aortic sinus of Valsalva are rare The purpose of presenting this paper is to report a case of the extremely rare aneurysms originating from the left aortic sinus This aneurysm ruptured resulting in acute myomalacia because of compression of the adjacent left coronary artery

Furthermore it is the purpose to draw attention to the fact that in certain cases surgical treatment of aneurysms of the sinuses is possible and consequently an early diagnosis is of the greatest importance Previously the diagnosis was rarely made *in vivo* but during recent years this has been done increasingly and in such cases primarily by roentgenological studies (2 16) Since 1957 aneurysms of the sinuses have been operated on successfully employing extracorporal circulation (11 15 19) *Aletras et al* (1963) have published a collective review of 23 successful operations

CASE REPORT

The patient was a 76 year old woman wh had been healthy previ u ly Ten days before admission she was taken ill with a severe attack of sten c rdia ccompanied by dyspnoea She was admitted because of cardiac in ufficien Th al examina tion on admission revealed pronounced dy pn ea at rest and mark d distension of jugular veins but no cyanosis Blood pressu 00 100 mg H Pulse rate 120 regular On auscultation the borders were t and 1 be at the fourth intercostal space and the left sternal border with the pr d impulse in the fifth intercostal space out ide the medioclavicular line The rhytm wa regular and n murmur could be detected with certainty Slight pretibial oedema was present Th remaining part of the physical examination revealed n rmal findings Electr cardiogram showed recent infarct of the anteri r wall of the eft ventricle ious tachycardia and ventricular extrasy t les Treatment was instituted with h pertonic glucose infusion morphine and oxygen whereby the dyspnoea wa hieved considerably A few hours later on the same da the patient again became extremely dyspnoic and now au cultati n revealed a systolic and diastoli ll wing murmur with its point of maximum intensity over the aorta Because of the e auscultatory findings

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For the permi sion to use the clinical material I am e pecially g at ful to Chief Physician *Jørgen Pedersen* M D Sønderbro Hospital Copenh gen Denmark

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it was presumed that perforation of the septum might have occurred. The above therapy was repeated successfully. During the subsequent days the patient's condition varied greatly. She was treated with digitalis and diuretics and in addition with anticoagulants because of thrombophlebitis of the left leg.

Other investigations: Chest roentgenogram revealed moderate cardiac enlargement, calcification of the aortic arch and moderate bilateral pulmonary congestion. Laboratory analyses: Haemoglobin 6.8 to 10.4 g per 100 ml; erythrocyte sedimentation rate 100 to 79 mm/h; red cell count 2.5 million per c mm; white cell count 14000 to 10000 per c mm. Serum lactic dehydrogenase 13.5 to 10.[illegible] mmol/h/l (normal range 3.4 to 9.8). Serum electrolytes normal.

Fourteen days after admission the patient's condition deteriorated. She became heavily dyspnoic and symptoms of bilateral bronchopneumonia developed. Consequently treatment with penicillin and theophyllamine was instituted, but the patient died the following day.

PATHOLOGICAL FINDINGS

The below description of the autopsy includes only such findings as are of interest in the present connexion. The heart weighed 600 g. On examination of the aortic orifice before the heart was opened pronounced stenosis was found. The cusps were thickened, rigid and inflexible. The diameter of the orifice was 4 mm and, because of calcification of the cusps, of irregular shape. Immediately above the semilunar valve an area measuring approximately 14 × 20 mm was found to be site of severe atherosclerosis towards which the stream of blood from the narrow aortic orifice had been directed (jet phenomenon). The remaining portion of the thoracic aorta presented moderate atherosclerotic changes only, whereas such changes were pronounced in the abdominal aorta.

The heart was opened by the commonly used method dividing the left semilunar cusp into two. The orifice of the left coronary artery was irregular and somewhat reduced in size because of atherosclerotic changes. An irregular opening in the aortic wall was found 3 mm below this orifice, about 5 mm in diameter, leading into a cavity approximately 2 cm long and 2 cm wide situated in the muscular part of the interventricular septum. This cavity was lined with a smooth shiny epithelium (Fig. 1) and filled with blood clots. At the apex of the cavity an irregular opening was seen, approximately 2 mm in diameter, through which blood from the aneurysm had penetrated into the adjacent muscular tissue in the interventricular septum (Fig. 2). The blood in the cavity maintained a certain pressure so that the aneurysm was seen on the surface of the heart to the left of aorta. Its position immediately below the left coronary artery caused the anterior descending branch to be forced upwards and its lumen to be narrowed. In the coronary artery there was a moderate diffuse atherosclerosis but no signs of thrombosis or emboli.

In addition, the wall of the left ventricle was somewhat hypertrophied, being up to 17 mm thick. Corresponding to the septum and to a small portion of the anterior wall of the left ventricle an area of the myocardium was streaked with haemorrhage and presented several



Fig 1
Section through anterior wall of the left ventricle A Peripheral portion of the aneurysm lined with endothelium and situated in the cardiac musculature closely related to and compressing B The anterior descending branch of the left coronary artery



Fig 2
Section corresponding to the septum A Wooden pin inserted from the left aortic sinus into the cross sectioned blood filled aneurysm B The anterior descending branch of the left coronary artery



Fig 3

Histological section from the septum. A The anterior descending branch of the left coronary artery B Elastic fibres corresponding to the wall of the aneurysm Furthermore cardiac musculature and connective tissue infiltrated by inflammation cells are seen (Weigert's elastin stain 400 ×)

small necroses The remaining valves and valvular orifices of the heart were normal

Microscopically in the zone surrounding the aneurysm there were extravasation of blood into the muscular tissue and pronounced infiltration of polymorphnuclear granulocytes in addition to more or less extensive areas consisting of necroses scattered between the muscular fibres (Fig 3) No signs of specific inflammation were present Neither were there any peri arterial changes or foreign body giant cells

DISCUSSION

The aortic sinuses of Valsalva derive their names from the coronary arteries arising from them. Hence there is a right, a left and a non coronary sinus. Aneurysms from these sinuses and in particular from the left sinus are rare. Since the first report on aneurysms of the aortic sinuses appeared (*Thurnam* 1840) the number of cases published has increased to more than 150. Several authors report fairly identical frequencies, i.e. approximately one case per 1000 autopsies (5, 6, 7). Among 5896 cases of aortic aneurysms *Snyder* (1937) found 0.93 per cent aneurysms of the sinuses. In an analytical study of 2000 cases of congenital heart disease 4 aneurysms from the aortic sinus were found (21). Aneurysms from the right and the non coronary sinuses are much more frequent than those from the left. Among 49 aneurysms 34 originated from the right, 13 from the non coronary and 2 from the left sinus (15). On review of the literature *Falholt & Thomsen* (1953) found similar frequencies. Ruptured aneurysms are stated to occur four times as frequently in males as in females. The average length of life is stated to be not more than 33 years (3).

The aetiology of the aneurysms has been the subject of discussion. To day it is believed that the cause stated by *Edwards & Burchell* in 1957 is contributory. They showed that the cause was a deficient communication between the media of the aorta and the annulus fibrosus. This explanation agrees well with the fact that most aneurysms are congenital (9). The non congenital aneurysms are caused mainly by atherosclerotic or syphilitic changes. Furthermore subacute or ulcerous endocarditis may be a contributory cause (11, 22), and finally cases have been reported in which presumably congenital aneurysms ruptured because of endocarditis (12).

Aneurysms from the right sinus rupture frequently into the right ventricle, the rupture from the non coronary sinus is almost always into the right atrium (8), and in case of a rupture of an aneurysm from the left sinus this will be into the pulmonary artery, the left ventricle or, as in the case reported in the present paper, into the epicardium and/or the myocardium (10, 13, 14). In cases of aneurysms from the right sinus there will often be a ventricular septal defect (15, 16).

Most commonly a non ruptured aneurysm is symptom free, in many cases the rupture is provoked by physical exertion (17, 18) and as a rule alarming symptoms will be present: severe precordial pain, dyspnoea and shock (9, 17). On auscultation a systolic and diastolic murmur will often be found resembling that heard in patent ductus arteriosus (23). In case of rupture death may occur within seconds although survival for up to 17 years after the rupture has been observed (11, 18). The most common course will be increasing cardiac insufficiency and death within a few months (10).

It has not been possible to decide with certainty whether the case reported in this paper is a genuine (congenital) aneurysm or a pseudo aneurysm Probability strongly supported the latter presumption since the aneurysm must be considered to have arisen in consequence of a weakness of the wall of the aorta because of the local but very pronounced atherosclerosis As regards both the mode of origin and the epicardial extension the aneurysm presents certain points of resemblance with one of the cases reported by *Sorensen & Kolsaker* (1962) In their case the coronary artery was not affected and the aneurysm had ruptured into the left ventricle In the available literature three cases were found in which an aneurysm from the left sinus or from the immediate surroundings of the latter had caused compression of the left coronary artery (2 10 14)

SUMMARY

A case of an aneurysm arising from the left aortic sinus of Valsalva is presented which resulted in occlusion of the anterior descending branch of the left coronary artery causing myocardial infarction The aneurysm had burrowed downward into the muscular part of the interventricular septum and corresponding to the distal part of the aneurysm rupture and diffuse extravasation of blood into the cardiac musculature were found The finding was interpreted as a pseudo aneurysm arising because of severe atherosclerosis and weakness of the wall of the aorta The importance of an early diagnosis and if possible surgical intervention in cases of aneurysms is mentioned

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A CASE OF INTRACEREBRAL CHONDROMA

A Case Report

By

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Received 16 x 68

Intracranial chondromas are rare occurrences Most usually they are found at the base of the skull where they may originate in the cartilaginous remains of the basal bones or in the nasal cartilages (*Klinger* 1951) Another common location is the meninges especially falx cerebri (*Russel* 1950) As a rule solitary intracranial chondromas have benign histological and growth patterns and in the majority of cases there is a clinical history of some years Headache dizziness epileptic attacks or convulsions are frequently present Usually symptoms disappear after extirpation of the tumour

True intracerebral chondromas are extremely rare Table 1 presents six such cases including the present one compiled from the previous literature Chondromas of obvious meningeal origin are excluded The case presented here was unusual in the sense that it was found at a medico legal autopsy following unexpected death Moreover the localization of the tumour diverged from that otherwise seen in most of the reported cases of intracranial chondroma

THE CASE HISTORY

Anamnesis

The man exposed to autopsy was a male 53 years of age The man had been operated upon 15 years ago for a perforated ventricular ulcus 19 years ago a foal had kicked him in the head and unconsciousness and convulsions followed The patient was observed in a hospital for two weeks but no serious injuries were reported About half a year later he had a sudden attack of unconsciousness as a result of subarachnoidal haemorrhage and the patient was examined in the Neurosurgical Clinic of the University of Helsinki A great calcified tumour locating in the midline above the turkish saddle was found The cerebral ventricles were dilated The tumour was regarded as inoperable due to its location and great size The patient was sent home where he was able to work with the exception of a few episodes of headache he was symptomless for the following 11 years According to the police report the man was found dead in his lonely cabin in the countryside Since the circumstances were unclear and death at the first sight seemed unexpected a medico legal autopsy was performed

TABLE 1

Some Cases of Intracerebral Chondromas Compiled from the Literature are presented together with the Case presented here. Chondromas Apparently of Meningeal Origin are not Included

Case	Sex	Age	Localization of the tumour	Size (diameter)	Main symptoms	Type of tumour	Reported by
1	♂	27	Choroid plexus III ventricle	2 cm	Headache, vomit, sudden death	Hyal. cart.	*Fell rer* 1970
2	♂	?	Choroid plexus lateral ventr.	2 cm	Epileptic attacks	Hyal. cart.	*Fell rer* 1920
3	♀	43	Left parieto-occip. region	?	Visual, audit. hallucinations	Fibrous cart.	*Jacob et al.* 1933
4	♂	64	Right temporal lobe	6 cm	Headache, dizziness, epileptic attacks	Primit. cart. gliomatous	*Rubinstein* 195[illegible]
5	♀	13	IV ventricle	great	Vomiting, soreness of neck	Hyal. cart. ependymous gliomatous	*Siqueira et al.* 1966
6	♂	53	[illegible] of lateral ventricle	4 cm	Not known, sudden death	Hyal. cart.	Own case

Autopsy Findings

The nutritional status of the deceased was good No signs of violence were found The ventricle had been resected and connected to the ileum Other pathological findings in the viscera were not observed

Apart from the neoplasm the brains were macroscopically normal The ventricles did not seem dilated In the left lateral ventricle a tumour 4 cm in diameter was attached to the floor by a thin short stalk but otherwise it was bathed freely in the cerebrospinal fluid The choroidal plexi were intact No haemorrhages were observable The surface of the tumour was rough and cauliflower like The consistency was firm and elastic and cartilaginous to the touch

Histological Examination

The specimen had to decalcified before sectioning This was effected in 1 7 per cent EDTA solution followed by embedding in paraffin The sections were stained with Haematoxylin Eosin PAS Alcian blue Cresyl fast violet and Toluidine blue for histochemical characterization

The specimen consisted of partially necrotic cartilaginous tissue (Fig 1) In the non necrotic areas the cells were typical chondrocytes in their lacunae The cells were large round and uniform The lacunae were surrounded by amorphous substance and by some fibres The ground substance in the non necrotic areas stained strongly metachromatically with Toluidine blue and Cresyl fast violet and was also PAS and Alcian blue positive The fibres were also PAS positive and stained weakly metachromatically (Fig 2) The necrotic areas lacked chondrocytes but exhibited a few macrophages and fibroblasts No metachromasy PAS or Alcian blue positivesness was discernible in these areas No calcification existed after EDTA treatment The tumour was surrounded by a thin connective tissue capsula No infiltrative growth through the capsula was observable

COMMENTS

From Table 1 it appears that intracerebral chondromas most frequently are located in the cerebral ventricles The most usual clinical symptoms have been headache vomiting and epileptic attacks The true chondromas have been benign in character but the mixed tumours with ependymalous or gliomatous components have exhibited also malignant features In the present case the death was preceded by the rather silent and symptomless period of 11 years and hence the sudden death came rather unexpected However the chondroma was considered as the only cause of the death since possible causes such as fresh haemorrhage and other acute signs were not observable

The investigated tumour consisted of cartilaginous tissue alone Since the chondrocytes were large uniform and surrounded by an appreciable

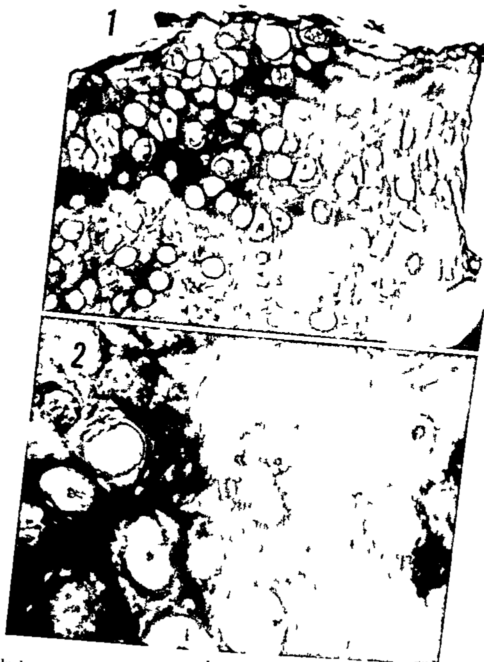


Fig 1

Toluidine blue 200 × In the upper edge typical chondromatous tissue which stains metachromatically In the lower half degenerating chondrocytes and a few fibroblasts are present Note the lack of metachromasy in the latter area

Fig 2

Cresyl fast violet 600 × To the left a few living chondrocytes which have secreted the metachromatic material To the right the degenerated tumour tissue

amount of ground substance and a few fibres which is in accord with the histological features of hyaline cartilage and since the tumour was not attached to the surroundings and no infiltrative growth existed the tumour was to be regarded as benign Histochemical characterization of the ground substance around the chondrocytes revealed the metachromatic staining and PAS and Alcian blue positiveness which point to acid mucosubstances possibly a chondroitin sulphuric acid content The necrotic areas lacked cartilage cells as well as the metachromatic ground substance

Unfortunately nothing but speculations can be made concerning the origin of the tumour The present chondroma noticeably resembled those reported by *Letterer* (Numbers 1 and 2 in Table 1) The three tumours were found in men had consisted only of chondromatous tissue had displayed solitary growth within the cerebral ventricles and were well separated as an exception no attachment was noticeable at the choroid plexus in the present case Thus in this case the meningeal and choroidal mesenchyme can be excluded as the site of metaplastic change In the light of the simultaneous existence of chondroma and ependymoma tissue within one tumour in case 5 in Table 1 it might be suggested that the present tumour originated in the ependyma of the lateral ventricle This seems very unlikely as no ependymatous cells existed and the tumour was thoroughly firm in consistency Consequently there remains the possibility of a metaplastic origin in the perivascular mesenchymal tissue beneath the ependyma The trauma caused by the kick to the head which occurred 12 years before the death and half a year before the finding of the great calcified tumour in the diencephalon might have been the factor that stimulated the proliferation of the mesenchyme The growing out and calcification should however have progressed at a very great rate It can neither be excluded that the tumour already existed at the time of the injury In this case the tumour should have originated *sui generis* An origin of this kind has also been previously suggested in connexion with the intracranial chondromas reported in the literature (*Letterer* 1920 *Chorobski et al* 1939)

SUMMARY

A case of intracerebral solitary chondroma is described It was found at the medico legal autopsy following the sudden death of a man of 53 The tumour was located in the left lateral ventricle attached to the floor and well separated The histological picture resembled hyaline cartilage partly in necrosis No malignant features were observed A list is also given of five intracerebral chondromas reported previously

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CELL RENEWAL OF THE NORMAL MOUSE CORNEA

By

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Received 31 x 68

Recent findings have given new information about the cell renewal in squamous epithelia According to the old concept the cell divisions in the basal layer result in one new basal cell and one differentiating cell Several new investigations indicate that this model is incorrect (*Leblond Greulich & Pereira* 1964 *Greulich* 1964 *Iversen Bjerknes & Devik* 1968) What actually seems to happen is that most cell divisions give rise to two new basal cells that both are potentially capable of dividing again But as new cells are formed in the basal layer other cells in this layer probably the oldest ones (*Iversen et al* 1964) are squeezed out into the layer of differentiating cells When the tissue is in a steady state the number of cells leaving the basal cells layer is on the average equal to the number of cells formed by the cell divisions

The new data cited above have been obtained by examination of the oesophageal epithelium (*Leblond et al* 1964 *Greulich* 1964) and of the mouse epidermis (*Iversen et al* 1968) The aim of the present study was to see if the normal cell proliferation pattern of another stratified epithelium is in conformity with these findings Thus the mouse cornea was examined by means of autoradiography after continuous labelling with tritiated thymidine (^{3}H T) and by the Colcemid method The results indicated that the new concept about the normal pattern of cell renewal in squamous epithelia is valid even in this tissue

METHOD AND MATERIAL

Hairless mice hr hr about 3 months old were used in all experiments The cornea of the mouse is comparatively great and comprises about the anterior third of the eye The central part of the corneal epithelium has 3–5 layers of epithelial cells At the limbus the epithelium is thicker and more mitoses are usually found here than in the central part The epithelium is of ectodermal origin but keratinization is never seen in the normal corneal epithelium

The ^{3}H T was injected intraperitoneally at 4 hour intervals for 10 days Each injection contained 10 mCi ^{3}H T in physiological saline The last group of mice that was killed after 10 days had thus received 600 mCi ^{3}H T The animals were killed by neck fracture and the eye removed and fixed in Bouin's solution for

24 hours After routine dehydration and paraffin embedding the specimens were cut in 5 μ sections Autoradiographs were made according to the stripping film method using Kodak 10 AR film plates After a 4 week exposure period in the refrigerator the films were developed and the sections stained with haematoxylin Labelled and unlabelled basal and differentiating cells were counted 1 000 cells in the anterior part of the cornea avoiding the thicker peripheral zone Cells with their nucleus oriented vertically on the basement membrane and with their cytoplasm in contact with the basement membrane were counted as basal cells Cells with flattened nucleus and with cytoplasm not in contact with the basement membrane were counted as differentiating cells The background activity being low cells with 3 grains or more were registered as labelled

The animals examined by means of the Colcemid method were injected with 0 15 mg Colcemid CIBA" intraperitoneally at 8 a m and killed exactly 4 hours later The eyes from these mice were treated in the same way as those in the previous series and the sections were stained with haematoxylin eosin and saffron Mitoses as well as basal and differentiating cells were counted separately Here too the peripheral zone of the cornea was avoided The mitotic rate (defined as the percentage of cells entering into mitosis per hour) was calculated by means of the equation $R = \frac{C}{t}$ where R is the mitotic rate and C the number of arrested mitoses t hours after the injection of Colcemid (for discussion of the method see Figsli & Dustin 1955 Figjo 1966)

EXPERIMENTS AND RESULTS

A *Tritiated Thymidine*

Groups of 4 animals were killed daily at 9 00 a m The first group was killed one day after the first injection of ^{3}H T and the last group after 10 days

The results are shown in Fig 1 The percentage of labelled basal cells increased rapidly during the first 2 3 days After 4 days the percentage of labelled basal cells showed only minor variations around the highest labelling values (94–95 per cent)

After a delay of about 1 day the percentage of labelled differentiating cells increased rapidly too It flattened out at about 4–5 days after the beginning of the experiment but even after the 5th day the percentage of labelled differentiating cells continued to rise slowly up to the 9th day In this series the basal cells constituted 44 per cent and the differentiating cells 56 per cent of the total cell population

B *Colcemid*

Six animals were injected with Colcemid as described above From these animals only 8 eyes could be used The remaining 4 eyes showed chronic inflammatory changes with hyperplasia of the epithelium and were therefore discarded

The results are shown in Table 1 The average number of arrested mitoses 4 hours after the injection of Colcemid was 20 ± 2 45 per 1 000 cells The average mitotic rate among the basal cells which in this series constituted 46 8 per cent of the total cell population is accordingly $\left(\frac{2 \times 100}{46\ 8 \times 4} \right) = 1\ 04$ per cent per hour The estimated mean generation time of the basal cells was $\left(\frac{100}{1\ 04} \right) = 97$ hours ($\approx$ 4 days)

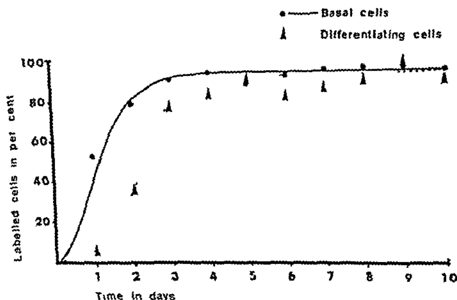


Fig 1

Autoradiographic labelling indices of basal and differentiating cells of the mouse corneal ephithelium ^{3}H T was given at 4-hour intervals for 10 days Groups of 4 mice were killed daily at 9 a m

TABLE 1

Percentages of Basal Cells Differentiating Cells and Mitoses 4 Hours after Injection of 0 15 mg of Colcemid

Number of eyes	Basal cells	Differentiating cells	Mitoses
8	46 80 ± 1 23	51 20 ± 1 17	2 00 ± 0 25

As the ratio between the differentiating cells and the basal cells in this series was on the average 1 09 the turnover time of the differentiating cells would be (97 × 1 09) = 106 hours (≈ 4–5 days)

DISCUSSION

The cell renewal of the normal corneal epithelium of various animals has been studied both by autoradiography after pulse labelling with ^{3}H T (*Hanna & O Brien* 1960) and by means of colchicine (*Buschke et al* 1943) *Hanna & O Brien* (1960) found that the turnover time of the mouse cornea was about 6–7 days —Their estimate was not founded on cell counts but on the observation that most labelled differentiating cells were sloughed off by that time *Buschke et al* (1943) have made extensive investigations on the rat cornea by means of the colchicine method These authors counted mitoses in a defined area of sheet

preparations of the corneal epithelium Their results are therefore difficult to compare with those obtained in the present study

The mitotic rate of the corneal epithelium is quite sensitive to various stimuli Adrenalin or excitement alone depress the mitotic rate (*Friedenwald & Buschke* 1944) while estrogens increase the rate of cell proliferation (*Epifanova* 1966) Most local anaesthetics also depress the mitotic rate and other even increases the mitotic duration (*Buschke et al* 1943 *Smelser & Ozanics* 1945)

A defect in the corneal epithelium is initially covered by epithelial cells that migrate over the denuded area The rise in the mitotic rate occurs after the closure of the defects (*Friedenwald & Buschke* 1955) It is not known however whether a similar migration of epithelial cells normally take place from the peripheral to the central part of the cornea

In the present study the mean generation time of the basal cells was about 4 days when calculated by means of the Colcemid method On the basis of this result it would be expected that the percentage of labelled basal cells should not reach its maximum till 4 days after the beginning of the experiment Actually the percentage of labelled basal cells attained its maximum of about 93 per cent by the 3rd day This finding implies that all cells in the basal layer had been through a DNA synthesizing phase at least once during the first 3 days of the experiment Thus there is a discrepancy between the mean generation time of 4 days estimated with the Colcemid method that of 3 days found by continous labelling with ^{3}H T This discrepancy can probably be explained on the basis of the new findings mentioned in the introduction

The equation for estimating the mean generation time of the basal cells with the Colcemid method implies that 100 per cent of the basal cells are continually dividing at certain intervals According to the new concept cited above only a certain number of the basal cells will divide again The rest of the cells in the basal layer will eventually be squeezed out into the differentiating layer The actual growth fraction is thus represented only by a certain number of the basal cells This growth fraction is not known but as it must be less than 100 per cent of the basal cells the real mitotic rate of this growth fraction will be higher and the mean generation time shorter than the values estimated by means of the Colcemid method The mean generation time of about 3 days found by the ^{3}H T method is therefore probably more correct This would mean that about 75 per cent of the basal cells belong to the progenitor cells population in the corneal epithelium

Fig 1 strongly supports the assumption that most cell divisions result in two cells that both remain in the basal layer for some time before one of them on the average is pushed out among the differentiating cells In the central part of the mouse cornea the number of basal cells is almost equal to the number of differentiating cells If

each mitosis had resulted in one basal cells and one differentiating cell the percentage of labelled differentiating cells would have increased parallel to that of the labelled basal cells In Fig 1 there is a delay of about 1 day before the percentage of labelled cells begins to increase This finding indicates that in the corneal epithelium too the main part of the divisions yields two new basal cells

In the ^{3}H T experiment the percentage of labelled differentiating cells increased rapidly from the 1st to the 4th day After this steep rise it continued to increase slowly for nearly the whole experimental period It is therefore difficult on the basis of the data to determine exactly the turnover time of the differentiating cells It is evident however that the greater part of the old cells were sloughed off during the first 4–5 days and substituted by new labelled cells But the last slow rise in the percentage of labelled differentiating cells suggests that some cells remain for quite a long time in the differentiating layer while other cells disappear after a shorter delay

It is probable that the mice used in the ^{3}H T experiment were stressed by the frequent injections As mentioned above adrenalin or excitement can depress the mitotic rate of the corneal epithelium (*Friedenwald & Buschke* 1944) The mean generation time could therefore be even shorter than 3 days in untreated animals However the growth parameters of the epidermal cell population of these animals appeared not to be significantly influenced by the repeated injections of ^{3}H T (see *Iversen et al* 1968 who examined the epidermis of the very same animals that were used in the present study)

Thus it can probably be concluded that the mean generation time of the progenitor part of the basal cells of the normal corneal epithelium is about 3 days The pattern of cell renewal of this tissue seems to conform well with that found in the oesophageal epithelium (*Leblond et al* 1964 *Greulich* 1964) and in the epidermis (*Iversen et al* 1968) As this cell renewal pattern implies that only a part of the basal cells are going to divide again the Colcemid method will not directly give information about the various parameters The size of the progenitor cell population must also be known

SUMMARY

The cell renewal of the normal mouse cornea was studied by means of autoradiographs after continuous labelling with tritiated thymidine and by the Colcemid method Continuous labelling was achieved by repeated injections of tritiated thymidine at 4 hour intervals for 10 days After continuous labelling in this way there was a delay of about 1 day before labelled cells appeared among the differentiating cells This probably means that most cell divisions in the basal layer result in two new basal cells that both remain in the basal layer for some time before one of them on the average is pushed out into the

differentiating layer This implies that only a certain part of the basal cells belongs to the progenitor cell population proper The thymidine experiment suggested that the mean generation time of these progenitor cells is about 3 days The turnover time of the differentiating cells was more difficult to determine exactly but the greater part of the differen tiating cells became labelled between the 1st and the 4th day Estimates made by means of the Colcemid method gave a lower mitotic rate and a longer mean generation time of the basal cells than found by the con tinuous labelling technique This discrepancy can probably be ex plained by the fact that the equations used in the Colcemid technique presuppose that all basal cells will divide again

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THE STABILITY OF THE EPIDERMAL MITOSIS INHIBITING FACTOR (CHALONE) IN WATER SOLUTION

By

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Received 23 x 68

Recent investigations have shown that aqueous extracts of epidermis contain a tissue specific but species unspecific mitosis inhibiting factor named chalone (*Bullough* 1962 *Bullough et al* 1967) It is assumed that it is produced by the keratinocytes and regulates the rate of cell renewal in the basal cell population according to a negative feedback principle (*Bullough* 1962) In a preliminary attempt at isolation *Bullough et al* (1964) found that a simple extract of mouse epidermis in a water solution loses its activity within several days at about 0 C and within several weeks at —20 C Moreover these experiments revealed that the activity of lyophilized extracts can be preserved for up to 12 months Later *Hondius Boldingh & Laurence* (1968) have tried to purify and concentrate the active fraction of pig skin chalone According to their findings chalone is a protein or a glycoprotein which is quite stable at a low pH and with a molecular weight of 30 000–40 000

Purification and concentration of chalone as performed by *Hondius Boldingh & Laurence* (1968) is difficult and can only be done in specially equipped laboratories Accordingly most experiments concerning the chalone effect must still be performed with simple aqueous skin extracts with or without subsequent freeze drying The aim of the present study was to examine the stability of the primary extracts The results clearly demonstrate that a water solution of lyophilized mouse skin extracts rapidly loses its mitosis inhibiting capacity at 37 C After 30 min at this temperature the extracts have no effect on the mitotic rate when tested *in vivo* on the hairless mouse epidermis

MATERIALS AND METHODS

Chalone containing extracts were made from the skin of hairless mice (hr/hr) After killing the skin was flayed off and cut to small pieces that were immediately put into a vessel surrounded by solid carbon dioxide When skins from about

My grateful thanks are due to *Mrs W Edgehill* for valuable technical assistance in this work

20 mice had been obtained in this way distilled water was added and the pieces homogenized in a Turrax Ultra homogenizer for about 5 min at 0 C. The homogenate was frozen in liquid air and ground to a fine powder in a porcelain mortar. The frozen powder was allowed to melt and was then centrifuged for 20 min at 25 000 × g and at 0 C. The clear supernatant was withdrawn and lyophilized. Using this procedure each mouse yielded about 30 mg lyophilized powder. This substance was used as chalone. It was stored at 0-4 C.

In the experiments the lyophilized powder was dissolved in distilled water at a concentration of 10 mg per ml. The pH of the solution was 7.65.

Fresh pig liver extracts were used as controls to test the effect on the epidermal mitotic rate of injections of some non-keratinizing epithelial tissue. The liver tissue was treated in exactly the same manner as the skin. Here too the lyophilized powder was dissolved in distilled water at a concentration of 10 mg per ml.

The activity of the various fractions were studied *in vivo* on the back skin epidermis of the hairless mouse (hr/hr). The rate of cell proliferation of the epidermal cell population was estimated by means of the Colcemid method. After intraperitoneal injection of 0.15 mg of Colcemid epidermal mitoses are arrested during the subsequent 4 hours. The number of mitoses accumulated during this period therefore gives an estimate of the number of cells beginning their division per unit time in a given cell population. In the present study mitoses arrested by Colcemid in 4 hours were counted in 8 mm interfollicular epidermis. (For further discussion of the method and details of the experimental procedure see Elgjo 1966.) In all experiments Colcemid was injected at 9.30 a.m. and 0.50 ml (5 mg lyophilized powder) of the fraction to be tested was given intraperitoneally 30 min later.

Experiments

Chalone solution. One part of the chalone-containing solution was injected immediately after preparation. Another part was stored at 37 C and fractions were removed for testing 0.25, 0.50, 1.00, 2.00, 12.00 and 24.00 hours after incubation. A third part was filtrated through a Millipore filter (pore size 0 μ) immediately after preparation and incubated at 37 C for 24 hours.

Liver solution. One part of the solution containing lyophilized liver extract was tested immediately after preparation. Another part was stored at 37 C and tested after 2.00 hours. A third part was filtrated through a Millipore filter immediately after preparation and stored at 37 C for 24 hours.

TABLE 1

Water Solutions of Lyophilized Aqueous Extracts of Skin or Liver Kept at 37 C for Various Periods of Time and Tested for their Effect on the Mitotic Rate of the Mouse Epidermis

Age of dissolved extract	Skin extract N	Skin extract Number of mitoses	Liver extracts N	Liver extracts Number of mitoses
Controls	31	23.10 ± 1.28	31	23.10 ± 1.28
0 hr	8	7.00 ± 0.79	8	22.00 ± 1.90
0.25 hr	4	14.25 ± 1.92		
0.50 hr	8	22.75 ± 1.22		
1.00 hr	8	21.37 ± 4.05		
2.00 hr	8	18.50 ± 2.13	[illegible]	19.50 ± 1.58
12.00 hr	[illegible]	17.50 ± 2.18		
24.00 hr	12	11.58 ± 1.43	[illegible]	(24.75 ± 1.91)
24.00 hr (sterile)	8	(23.50 ± 1.42)		

Each animal was given 5 mg of extract in 0.5 ml of distilled water i.p. 30 min after injection of Colcemid. The number of mitoses arrested by Colcemid in 4 hr is given per 8 mm interfollicular epidermis together with the standard error of the mean. The results of filtrated sterile extracts are in brackets.

RESULTS

Table 1 shows that 5 mg of chalone depressed the mitotic rate to about 30 per cent of the normal value when administered immediately after being dissolved in water. After 15 min at 37° C the chalone solution still depressed the mitotic rate by about 50 per cent. The solutions that had been kept for 0.50, 1.00 and 2.00 hours respectively had no significant effect on the mitotic rate ($p<0.05$). The unfiltered solutions that had been incubated for 12.00 or 24.00 hours depressed the rate significantly ($p<0.05$), while the filtrated 24 hour old solution had no effect on the mitotic rate.

The various liver extracts did not influence the mitotic rate significantly ($p<0.05$).

DISCUSSION

Nothing is as yet known about the production or the metabolism of chalone. It is likely that it would be rapidly metabolized *in vivo*. From a theoretical point of view this should be a prerequisite of a chemical signal in a cybernetically regulated system such as the epidermal cell population. The breakdown of chalone could be brought about by a special enzyme system, or it might be "used up" by being bound to specific sites in the progenitor cells.

Table 1 shows that the activity of the lyophilized skin extracts decreased gradually and almost proportionally to the incubation time during the first 30 min. This degradation was accomplished in a cell free solution. The results therefore indicate that the mitosis inhibiting principle was broken down enzymatically.

The mitosis inhibiting effect of the two old chalone solutions was evidently unspecific and due to bacterial contamination, as the sterile 24 hour old solution had no effect on the mitotic rate. On autopsy of the mice injected with the two old solutions that had not been filtered it was found that the peritoneal surface was inflamed and some fluid was usually present in the peritoneal cavity. The animals injected with chalone solutions not more than 2 hours old, with sterile 24 hour old chalone solution, or with liver extracts did not show any signs of peritoneal irritation.

The conclusions that can be drawn from the presented data can be summarized as follows:

1. When aqueous solution of skin extracts are to be tested for their mitosis inhibiting effect it is of utmost importance that they should be used immediately after being dissolved in water.
2. The temperature should be kept as low as possible through all stages of the preparation of the skin extracts that are to be tested for their effect on the mitotic rate.
3. Old solutions should be discarded if they are unsterile as they may depress the mitotic rate in some unspecific way.

SUMMARY

Water solutions of lyophilized aqueous extracts of mouse skin were kept at 37° C and tested for their mitosis inhibiting effect after various incubation times The activity of the extracts was tested on hairless mouse epidermis by means of the Colcemid method The water solution depressed the epidermal mitotic rate significantly when injected i p immediately after being dissolved in water After 30 min at 37 C all activity was lost This indicates that the epidermal mitosis inhibiting principle (chalone) is rapidly destroyed at 37 C in water solution

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GONADECTOMY AND TESTOSTERONE SUBSTITUTION IN MALE MICE WITH SPONTANEOUS ADRENOCORTICAL LIPID DEPLETION

A Comparison with C57Bl Mice

By

KÅRE MOLNE

Received 15 x 68

Adult mice of the AKR/O strain and the substrains CS and AC exhibit a spontaneous depletion of adrenocortical lipids as revealed by Sudan stained sections (1 3) The lipid depletion is almost total in adult males (Figs 1 and 2) whereas in females the depletion is subtotal with considerable variation from animal to animal This anomaly is due to the effect of a recessive gene called the adrenocortical lipid depletion gene (3) A short review of previous studies of these mice is given in another paper (8)

Cortisone treatment has indicated (2) and hypophysectomy experiments have shown (4) that this adrenocortical lipid depletion is dependent upon an intact pituitary function However gravimetric (8) and histometric (9) studies of the adrenal glands gave no evidence of an abnormal pituitary activity

Since the depletion of lipids from the adrenal cortex takes place at the time of sexual maturation (7) and since prepubertal gonadectomy partially prevents the development of the lipid depletion (1) the relationship between the gonadal function and the spontaneous adrenocortical lipid depletion was studied in gonadectomy and testosterone substitution experiments The results of this work are reported in the present paper

MATERIAL AND METHODS

Male mice of the AC strain were used This is a hybrid line derived from the AKR/O strain which is homozygous for the adrenocortical lipid depletion gene (3) Male C57Bl mice with a normal adrenocortical lipid pattern were used as controls

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I am indebted to the head of the Life Insurance Companies Institute for Medical Statistics at the Oslo City Hospitals, dr *Knut Westlund* for his helpful advice regarding the statistical analyses



Fig 1
Adrenal gland of AC male mouse 12 weeks old Spontaneous adrenocortical lipid depletion Sudan III × 33

Fig 2
Adrenal gland of C57Bl male mouse 12 weeks old. Normal adrenocortical lipid pattern Sudan III × 33

The animals were kept under standardized h u m and dietary conditions as described previously (8)

All experiments were performed when the anim ls were 11 12 weeks old At this age the animals are sexually mature and in th AC train the spontaneous adreno cortical lipid depletion is completed (1)

The material comprised 20 experimental gr ups with 10 animals in each group (Table 1) Gonadectomies and sham operations were perf rmed on gr ups of AC males and the animals were killed at int rvals ranging fr m 1 day to 6 weeks after the operation Other groups of AC and C57Bl animals were gonadectomized and received varying doses of testosterone in the post operative period All these animals were killed 3 days after the operation The experimental pr edure and body weight of all groups are given in Table 1

Operations The gonadectomies were performed thr ugh abd min l incisions under ether anaesthesia. Both the epidid ymide and the testes were removed In the sham operations the gonads were exp ed throu h lapar t my and then left intact

Testost rone substitution Te tosterone pr pi nate Te tin N (h solved in sesame oil) was given subcutaneously in the inter apular r g n twice daily The first injection was given at the end of th operati n and the la t inj ti n 1 h ur befo e sacrifice

Fixation and staining technique All animal w r acrifi d b cer i l disloca tion The adrenals were di sected free fr m fat ti u und r the di e tion micro scope and immersed in *Baker*s formaldehyde lutı n f r t w One adrenal gland was embedded in paraffin and secti ned at 3 mi r n The secti n were stained with haematoxylin and eosin Th th r ir n l gland wa embedded in gelatine and cut on the freezing microtome at 1 mi r n The sectı n were stained with Sudan III according to the method of *Daddi* de ribed by *Romeis* (10)

Evaluation of sections The paraffin secti n w r tudi d with regard t altera tions of histol gical architecture and cytol gi l det l

The Sudan stained sections were photo raph d under standard conditi ns with a Zeiss photomicroscope and a constant exp ure time Contact copie were made under standard conditi ns and marked with d numbers The ph t raphs were then ranked blindl int 7 cla ses according to the degree f adrenocortical Sudanophilia cla ificati n being made by the ni r member f th t ff (Anesen)

TABLE 1

The Experimental Procedures and Body Weight of 20 Groups of 11–12 Weeks Old AC and C57Bl Male Mice Each Group Contains 10 Animals

Strain	Operation	Survival after operation	Testosterone substitution (μg a day)	Body weight§ (Mean and range in g)	
AC	G	1 day	None	21.9	19.5–24.0
AC	S	1 day	None	22.6	20.0–24.0
AC	G	2 days	None	22.1	19.5–23.5
AC	S	2 days	None	23.2	20.5–25.0
AC	G	3 days	None	21.4	19.5–23.0
AC	S	3 days	None	22.4	19.5–25.0
AC	G	1 week	None	23.3	20.5–25.0
AC	S	1 week	None	22.2	20.5–24.5
AC	G	3 weeks	None	22.0	19.5–24.0
AC	S	3 weeks	None	21.8	20.0–23.5
AC	G	6 weeks	None	23.2	23.0–25.0
AC	S	6 weeks	None	22.1	18.0–24.5
AC	None	–	None	22.3	19.0–24.0
AC	G	3 days	12.5	22.6	21.5–24.5
C57Bl	None	–	None	24.6	22.5–2[illegible].0
C57Bl	G	3 days	None	24.3	20.0–27.0
C57Bl	S	3 days	None	24.[illegible]	20.0–27.5
C57Bl	G	3 days	12.5	23.7	[illegible]2.5–25.5
C57Bl	G	3 days	250.0	24.7	21.5–27.5
C57Bl	G	3 days	2500.0	24.6	21.5–27.0

G = Gonadectomy S = Sham operation
§ Recorded at the start of the experiment

In this rank scale the units 4–5 are considered to represent variations of a normal adrenocortical lipid content and the units 1–3 variation of lipid depletion Further information concerning this evaluation are given in another paper (7)

Statistical methods The results were evaluated statistically by the Student's t test and the Wilcoxon rank sum test The following significance levels were used $P > 0.05$, $0.01 < P < 0.05$, $P < 0.01$ When the t test and the rank sum test resulted in P values at different level both values have been given The term not significant refers to P values higher than 0.05

RESULTS

The adrenal cortex after gonadectomy Gonadectomized and sham operated animals were studied at intervals varying between 1 day and 6 weeks after the operation In *paraffin sections* of gonadectomized animals no alterations were found until 3 weeks after operation At this time a secondary X zone was found in 7 out of 10 animals This zone which is a well defined structure in gonadectomized adult male mice (6) is located between the permanent adrenal cortex and the medulla and consists of tightly packed cells with sparse and dark cytoplasm Three weeks after operation the secondary X zone had a thickness of 5–10 cell layers in the AC males Six weeks after gonadectomy the secondary X zone was present in 9 out of 10 animals and had a thickness of 20–25 cell layers The permanent adrenal cortex (zona glomerulosa and zona fasciculata) did not show any obvious

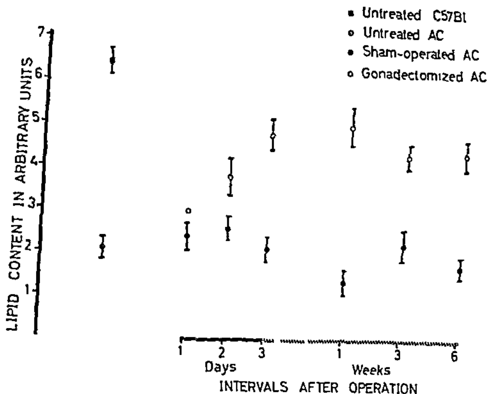


Fig 3

The adrenocortical lipid content of castrated and sham operated AC mice at different intervals after the operation The symbol designate the values for the arithmetic mean and standard error of the mean of each group Scale units 7–5 represent variations of a normal adrenocortical lipid content scale units 3–1 variation of lipid depletion Each group contains 10 animals

cytological changes in haematoxylin eosin stained section at any of the post operative intervals None of the sham operated animals developed a secondary X zone or other histological changes which could be identified in paraffin sections

The *Sudan stained sections* from gonadectomized and sham operated animals were evaluated semiquantitatively with regard to the adrenocortical lipid content and the results are given in Table 2 and in Fig 3

In gonadectomized AC males an increase of adrenocortical lipids took place from the first day after operation This adrenocortical repletion was evenly distributed throughout the entire permanent cortex Two days after gonadectomy the difference between gonadectomized and sham operated animals is significant on the 5 per cent level of probability (Table 2) and from the third day the adrenocortical lipid content seems to have reached a relatively stable level However this level is lower than the normal level of adrenocortical lipids in C57Bl males (Figs 1–4 and Tables 2 and 3) The difference between gonadectomized AC males surviving for one week and presenting the highest

TABLE 2

The Adrenocortical Lipid Content in Arbitrary Units of Gonadectomized and Sham Operated Adult AC Male Mice Each Group consists of 10 Animals

Survival after operation	Adrenocortical lipid content (arbitrary units)				Significance level of difference between groups (P)	
	Gonadectomy		Sham operation		Student s t test	Wilcoxon Rank Sum Test
	Mean	S D	Mean	S D		
1 day	3 0	0 0	2 4	0 82	0 01<P<0 05	0 5 <P
2 days	3 8	1 25	2 6	0 67	0 01<P<0 05	0 01<P<0 05
3 days	4 8	0 94	2 1	0 88	P<0 01	P<0 01
1 week	5 1	1 20	1 4	0 67	P<0 01	P<0 01
3 weeks	4 4	0 67	2 3	0 82	P<0 01	P<0 01
6 weeks	4 5	0 82	1 8	0 67	P<0 01	P<0 01

The scale ranges from 7-1 7-5 represent variations of a normal adrenocortical lipid pattern 3-1 variations of lipid depletion
S D Standard deviation

mean value of adrenocortical lipids and the untreated C57Bl mice is statistically significant (*Student* s t test P<0 01 Wilcoxon Rank Sum Test 0 01<P<0 05)

In the sham operated animals the adrenocortical Sudanophilia varies between scale units 1 and 3 which in our ranking system is characteristic for the lipid depleted adrenal cortex

In the C57Bl strain gonadectomy leads to a moderate *increase in* adrenocortical Sudanophilia after 3 days (*Student* s t test P<0 01 *Wilcoxon* Rank Sum Test 0 01<P<0 05) — Table 3 and Fig 5

The adrenal cortex of gonadectomized males with testosterone substition A postoperative survival time of 3 days was chosen for these series since gonadectomized AC males had reached a relatively stable level of adrenocortical lipids by this time The substitution doses of testosterone which were used are shown in Table 1

The *haematoxylin and eosin stained sections* did not reveal any obvious changes as regards adrenocortical histology in any of these



Fig 4

Adrenal gland of a 12 weeks old AC male mouse 3 days after gonadectomy Cortex partially repleted with lipid Sudan III × 33

groups. The results of a semiquantitative evaluation of Sudan stained sections are given in Table 3 and Fig. 5.

As regards adrenocortical Sudanophilia there is a clear strain difference when groups of untreated, gonadectomized or sham operated AC and C57Bl animals are compared ($P<0.01$).

TABLE 3

The Adrenocortical Lipid Content of Gonadectomized and Hormone Substituted AC and C57Bl Male Mice. Each Group Consists of 10 Animals. The Post Operation Survival was 3 Days

Experimental procedure	Adrenocortical lipid content (arbitrary units)	
	Mean	S.D.
AC no treatment	2.1	0.75
AC gonadectomy	4.9	0.94
AC sham operation	2.1	0.83
AC, gonadectomy testosterone substitution 12.5 µg daily	1.7	0.47
C57Bl no treatment	6.4	0.47
C57Bl gonadectomy	7.0	0.0
C57Bl sham operation	6.7	0.47
C57Bl gonadectomy testosterone substitution 12.5 µg daily	6.9	0.33
C57Bl gonadectomy testosterone substitution 250 µg daily	6.3	0.67
C57Bl gonadectomy testosterone substitution 2500 µg daily	5.7	0.67

The scale ranges from 7–1; scale units 7–5 represent variations of a normal adrenocortical lipid pattern, 3–1 variations of lipid depletion.
S.D.: Standard deviation.

In gonadectomized AC males 12.5 µg of testosterone a day in the post operative period prevents the lipid repletion; there is no significant difference between this group and groups of untreated AC males. According to *Solem* (11) this may be considered an adequate dose for testosterone substitution in adult mice.

The moderate increase in adrenocortical Sudanophilia in gonadectomized C57Bl male mice was prevented with 250 µg of testosterone daily in the postoperative period, while a daily substitution dose of 12.5 µg testosterone did not convincingly do so (difference in adrenocortical Sudanophilia: Student's t test $0.01<P<0.05$; Wilcoxon Rank Sum Test $0.05<P<0.1$). Using a daily dose of 2500 µg of testosterone the adrenocortical Sudanophilia was significantly reduced as compared with untreated animals of the same strain (Student's t test $0.01<P<0.05$; *Wilcoxon Rank Sum Test* $P=0.05$). However, in none of these animals did the adrenocortical Sudanophilia decrease to the level seen in untreated AC mice.

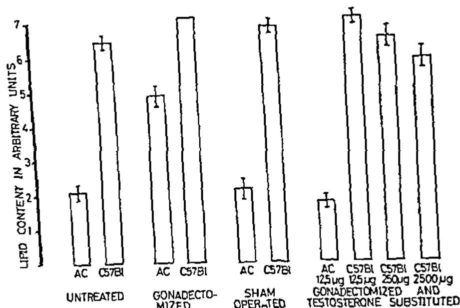


Fig 5

The adrenocortical lipid content in different groups of male AC and C57Bl mice The columns represent mean value and standard errors of the mean are indicated The arbitrary units are defined as in Fig 3

DISCUSSION

The present study has demonstrated that the spontaneous adreno cortical lipid depletion of AC male mice is dependent upon an intact endocrine function of the testes Gonadectomy of adult animals leads to a repletion of adrenocortical lipids within 3 days and the hormone substitution experiments showed that this effect was due to a cessation of testosterone production (Table 3 and Fig 5) It cannot be decided from the present experiments whether the testosterone acts directly on the adrenals or through a feed back mechanism *via* the pituitary

Although the increase in adrenocortical lipids in the AC males after gonadectomy was quite marked it did not bring the lipid content up to the level seen in the control strain (Table 3) Corresponding results were obtained by prepubertal gonadectomy in mice with spontaneous adrenocortical lipid depletion (1) Furthermore excessively high doses of testosterone in C57Bl males did not bring the lipid content down to that of the AC mice (Table 3) The spontaneous lipid depletion in AC males can therefore not be due to an abnormally high secretion of test osterone alone

A moderate increase in adrenocortical lipids was found even in the control strain after gonadectomy (Table 3) Similar findings have been made by *Chester Jones* (5) in another normal strain of mice The effect of gonadectomy may therefore be basically similar in AC mice and in

normal mice However the increase of adrenocortical Sudanophilia is much more marked in AC mice Analogous results have been obtained after hypophysectomy in AC mice (4)

SUMMARY AND CONCLUSION

Following gonadectomy on adult male mice with spontaneous adreno cortical lipid depletion (AC strain) lipid repletion takes place in the adrenal cortex Testosterone substitution prevents this lipid repletion In sham operated animals no alteration of the adrenocortical lipid pattern is found

A similar pattern of reaction is found in corresponding experiments with a control strain (C57Bl) but the accumulation of lipids in the adrenocortical cells of the C57Bl males after gonadectomy is not so marked as in mice with spontaneous adrenocortical lipid depletion Excessive doses of testosterone in C57Bl males does not result in a lipid depletion corresponding to that which occurs spontaneously in mice of the AC strain at the time of sexual maturation

It is concluded that the specific adrenocortical lipid pattern of male AC mice is dependent upon an intact endocrine gonadal function The mode of interaction is complicated and the present study indicates that the spontaneous adrenocortical depletion is not simply due to an in creased secretion of testosterone

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REGULATION OF OUTPUT OF LYMPHOCYTES FROM THE SPLEEN

1 *A Quantitative Investigation in Normal Sham Operated and Thymectomized Guinea Pigs*

By

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Received 2 xii 68

In a previous investigation the lymphocyte content in afferent and efferent splenic blood in guinea pigs was compared The results indicated a migration of lymphocytes from the spleen (*Ernstrom & Sandberg* 1968) The first aim of the present investigation was to study this emigration of splenic cells with differentiation of the lymphocytes into subclasses

In mice and rats neonatal thymectomy results in a deficiency of lymphocytes and defect immunological reactivity (*Arnason et al* 1962 *Martinez et al* 1962 *Miller* 1962) In animals more mature at birth and in adult animals thymectomy does not result in such pronounced morphological and immunological defects The influence of the thymus may be due to a thymic hormone necessary for normal growth of the lymphatic tissue and/or to a thymic export of lymphocytes populating the non thymic lymphatic tissue Such an export of thymic lymphocytes exists even in adult animals (*Nossal* 1964 *Murray & Woods* 1964 *Ernstrom et al* 1965 *Weissman* 1967 *Linna* 1968) A directed migratory stream of thymic lymphocytes to the spleen was postulated by *Fichtelius* (1953) and others Theoretically thymectomy may cause lack of some thymic humoral factor(s) and/or result in absence of a flow of thymic cells into the spleen perhaps leading to decreased production and emigration of splenic lymphocytes On the other hand some regulatory mechanism may result in an increased output of splenic lymphocytes compensating for the absence of circulating lymphocytes of thymic origin In the isolated rat spleen the release of cells is influenced by the number of leucocytes in the perfusate indicating such a regulation (*Dornfest & Piliero* 1966) The second aim of the

The expenses of this investigation were defrayed by a grant from the *Swedish Cancer Society*

present investigation was to study the influence of thymectomy on the export of splenic lymphocytes in guinea pigs

MATERIAL AND METHODS

The experiment included totally 119 male guinea pigs weighing 200–240 g at the start of the experiment The following experimental schedule was used

Normal guinea pigs (11 animals)

Thymectomized guinea pigs examined 1 2 4 8 16 32 and 64 days after thymectomy (11 15 10 10 10 12 and 13 animals respectively)

Sham operated guinea pigs examined 1 and 64 days after sham operation (15 and 12 animals respectively)

The thymectomies and sham operations were performed under local anaesthesia (Xylocaine® Astra 0 5 per cent) A cervical incision was made and the two thymic lobes were removed or mobilized respectively The wound was closed by silk sutures The procedure was essentially according to *Gyllensten* (1953)

On investigation the animals were anaesthetized with 2 5 per cent Nembutal sodium (25–50 mg kg b w i p) The peritoneal cavity was opened by an incision between the last two ribs on the left side One vein from the spleen was incised and blood was collected in a dry heparinized pipette (Heparin® Vitrum Stockholm Sweden) Immediately afterwards a splenic artery was incised and arterial blood collected in the same way

The blood samples were used for white cell counts in a Bürker counting chamber and for differentiation between polynuclear granulocytes and mononuclear cells of the white cells Furthermore a blood sample was used for supravital staining of the lymphocyte into subclasses

and for differentiation of the lymphocyte into subclasses

For supravital staining Janus green B (0 4 per cent) and neutral red (0 9 per cent) were dissolved separately in pure ethyl alcohol The actual mixture was freshly made before use and consisted of 1 part of Janus green B and 10 parts of neutral red solution This staining mixture was flooded over a clean slide which was then air dried A small drop of blood was applied to the slide and covered by a coverslip which was then ringed with paraffin wax to prevent evaporation After about 10 minutes the specimens were examined in a light microscope at a magnification of 1000 × In each preparation 100 lymphocytes were registered in six classes according to the cellular content of mitochondria cells with 0–5 6 10 11–15 16–20 21–30 and > 30 mitochondria A mitochondrial content of 0 10 11 20 and > 20 was denoted as low medium and high respectively As the mitochondrial content (MC) is correlated to the size of the lymphocytes low MC corresponds to small cells medium MC to medium sized and high MC to large cells (*Wiseman* 1931 *Lichtelius* & *Larsson* 1961 *Ernström* & *Larsson* 1963)

From the counts of mononuclear cells and the distribution of lymphocytes in the different subclasses the absolute number of lymphocytes per mm³ of blood from the splenic artery and the splenic vein was calculated The splenic veno arterial differences in the number of different white blood cells were obtained from the individual animals and the differences were analysed statistically by Student s *t* test

RESULTS

Normal Animals

In the normal non operated guinea pigs the lymphocyte content per mm³ of blood from the splenic vein exceeded that from the splenic artery (Fig 1)

A subdivision of the lymphocyte population based on the mitochondrial content of the cells resulted in the following veno arterious differences in number of cells per mm³ of blood passing through the spleen (M ± S E)

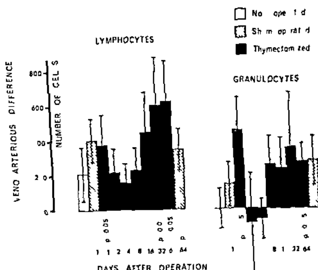


Fig 1

Differences between number of white cells per mm³ of bl l fr m a splenic vein and a plenic artery at different intervals after thymectomy and sham operation Mean ± S E

Lymphocytes with low mitochondrial content (small cells) 120 ± 88 (*i e* an increase in the venous blood of 20 per cent)

Lymphocytes with medium mitochondrial content (medium sized cells) 82 ± 67 cells (*i e* an increase of 17 per cent)

Lymphocytes with high mitochondrial content (large cells) 10 ± 3 cells (*i e* an increase of 71 per cent)

No difference between the number of granulocytes per mm³ of blood from the splenic vein and artery was found (Fig 1)

Sham Thymectomized Animals

Sham thymectomized animals were examined 1 and 64 days after sham operation At 1 day the lymphocyte number was similar to that in the normal non-operated animals At 64 days the lymphocyte number was much higher (Table 1) This is ascribed to the normal increase in growing guinea pigs (see *Ernstrom & Larsson* 1966)

The subdivision of the lymphocyte population showed more large lymphocytes and less small lymphocytes 1 day after surgery After 64 days all types of lymphocytes were increased in number (Table 1)

The splenic veno arterial difference in number of lymphocytes was larger than in the non operated animals but not significantly (Table 1 Fig 1)

The granulocytes were increased in number both 1 and 64 days after operation The first increase is an early granulocytosis seen after surgery and after administration of adrenal steroids (*cf Ernstrom & Lars*

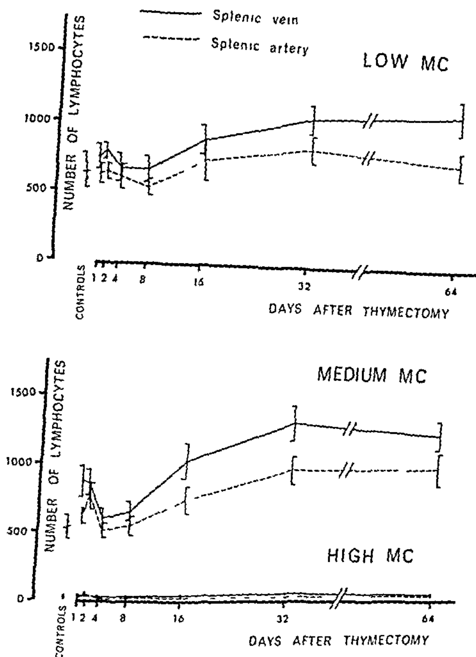


Fig 2

Number of lymphocytes per mm³ of blood from a splenic vein and a splenic artery at different intervals after thymectomy The lymphocytes are subdivided into cells with low medium and high mitochondrial content (MC) Mean ± S E.

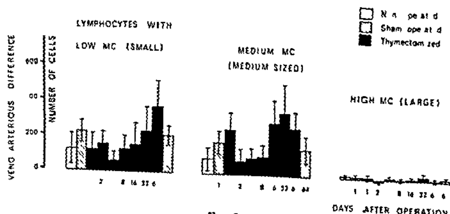


Fig 3

Difference between number of lymphocytes per mm³ of blood from a splenic vein and a splenic artery at different intervals after thymectomy and sham operation The lymphocytes are subdivided into cells with low medium and high mitochondrial content (MC) Mean ± S E

son 1967) The second increase seems to be an increase with age (*cf Ernstrom & Larsson* 1966)

Thymectomized Animals

Lymphocytes as well as granulocytes were increased in number 1 and 2 days after thymectomy in comparison with the values in the non operated animals After 4 days the number was about normal again Later after operation (16 32 and 64 days) the number of lymphocytes (Fig 2) and granulocytes was increasing As no significant differences between the values in sham operated and thymectomized animals were found the changes cannot be ascribed to a specific effect of thymectomy (Table 1)

The subdivision of the lymphocyte population showed that the changes in total lymphocyte number after thymectomy were reflected in all three main subpopulations of lymphocytes although they were most pronounced in the medium sized lymphocytes with medium mitochondrial content (Fig 2)

The splenic veno arterial difference in total number of lymphocytes was slightly increased 1 day after thymectomy but not to a significant level and did not differ from the increase in sham thymectomized animals The difference decreased in the period from 1 to 4 days From 4 to 64 days after operation the splenic veno arterial differences increased successively (Fig 1)

The subdivision of the lymphocyte population showed that early after thymectomy (1 8 days) the splenic veno arterious difference in number of lymphocytes of the smallest type with 0–5 mitochondria per cell was small in comparison to that in the non operated and sham

TABLE 1

Number of Lymphocytes and Granulocytes in Splenic Venous and Arterial Blood of Normal Guinea Pigs and of Thymectomized and Sham Operated Guinea Pigs 1 and 64 days after Operation

	Interval after operation	Number of lymphocytes per mm³								Number of granulocytes	
		Total no.		No. with low MC		No. with medium MC		No. with high MC			
		SV	SA	SV	SA	SV	SA	SV	SA	SV	SA
Thymectomized	1	1155 ±201	1278 ±110	753 ±88	633 ±69	872 ±119	624 ±51	30 ±7	21 ±[illegible]	1817 +264	1366 ±147
Sham operated	1	1499 ±133	108 ±107	683 ±76	461 ±53	763 ±60	594 ±51	36 ±5	30 ±4	1441 ±140	1298 ±174
Thymectomized	64	2420 ±213	1749 ±2[illegible]1	1179 +1[illegible]8	765 ±96	1237 +110	987 ±19	44 ±7	37 ±5	1956 ±187	1690 +210
Sham operated	64	2195 ±190	1857 ±197	998 +79	793 ±77	1139 ±112	1016 ±17	58 ±10	46 ±8	1963 ±193	1691 +162
Nonoperated		1309 ±118	1098 +99	714 +67	593 +57	572 +48	490 ±44	24 +3	14 +4	1048 ±95	1043 ±133

Mean no. of cells per mm³ of blood ± SE. SV = Splenic vein. SA = Splenic artery. No significant difference between corresponding thymectomized and sham operated animals was demonstrated.

TABLE 2

Effect of Thymectomy on the Splenic Veno Arterious Difference in Number of Lymphocytes Belonging to the Class Characterized by 0–5 Mitochondria per Cell (Smallest Lymphocytes)

	Days after operation						
	1	2	4	8	16	32	64
Thymectomized	7±19	7±12	8±21	—4±8	24±13	23±37	64±28
Sham operated	32±14						44±21
Non operated	28±19						

No per mm³ Mean Difference ± S E
Denotes significant export $p < 0.05$

operated (Table 2) A similar low output of small and medium sized lymphocytes was found 2–8 days after thymectomy Later after thymectomy the increasing veno arterious difference in total number of lymphocytes was due to an increasing difference in number of small and medium sized cells with low and medium mitochondrial content (Fig 3)

DISCUSSION

In a previous paper a splenic veno arterious difference of lymphocytes was reported (*Ernstrom & Sandberg* 1968) The present investigation has shown that the excess lymphocytes in the splenic veins compared to the artery belong to all categories of lymphocytes characterized by different mitochondrial content and different size This is in contrast to the export of lymphocytes from the thymus consisting exclusively of small cells with few mitochondria in their cytoplasm (*Ernstrom* 1965)

Thymectomy resulted in an increased number of lymphocytes and granulocytes in the blood after 1 and 2 days This was not however a specific effect of thymectomy as it was also observed in the sham thymectomized animals At 4 days the number of white blood cells was normal again At longer intervals after thymectomy the number of lymphocytes was successively increasing This seems to be the normal increase in number of small and medium sized lymphocytes occurring in growing guinea pigs (*Ernstrom & Larsson* 1966)

The splenic veno arterious difference in number of lymphocytes was slightly increased 1 and 2 days after thymectomy and decreased to normal again at 4 days As regards the smallest lymphocytes with 0–5 mitochondria per cell the output was diminished to subnormal level 4–8 days after thymectomy This decrease may be compared to a similar but more pronounced decreased output of splenic lymphocytes after combined thymectomy and steroid treatment (*Ernstrom & Sandberg* 1969)

At later intervals up to 64 days after thymectomy a successively in

creasing output was observed This increase consisting of small and medium sized lymphocytes may be a late effect of thymectomy but may also be an increase dependant on age The relation between the splenic output of lymphocytes and the age of the guinea pig will be further studied in a following investigation

SUMMARY

The lymphocyte population of blood from a splenic vein and a splenic artery was studied in normal intact guinea pigs in thymectomized and sham thymectomized guinea pigs The investigation was performed at intervals of 1 to 64 days after operation The number of lymphocytes and granulocytes per mm³ of blood was determined The lymphocyte population was divided into subgroups classified by their mitochondrial content which is correlated to the size of the lymphocyte Special attention was focused on the difference between lymphocyte number in the blood from the splenic vein and the splenic artery indicating migration of cells to or from the spleen The following observations were made

1 The exported lymphocytes from the spleen of normal young guinea pigs belong to all categories of lymphocytes characterized by different mitochondrial content

2 Increased output of splenic blood lymphocytes was observed 1 day after thymectomy followed by decrease to or below normal levels at 2–8 days after which a successive increase was found to the end of the experiment

3 An early increase in the output of lymphocytes was observed after sham operation as well as after thymectomy i e the increased export of white blood cells from the spleen shortly after thymectomy is most probably a non specific operative effect

4 The number of lymphocytes in the blood varied in a similar way as the splenic export of lymphocytes The increased number and output at later periods of the experiments may depend on increasing age of the experimental animals

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REGULATION OF OUTPUT OF LYMPHOCYTES FROM THE SPLEEN

2 A Quantitative Investigation in Sham Operated and Thymectomized Guinea Pigs During Steroid Induced Involution and Regeneration

By

ULF ERNSTROM and GORAN SANDBERG

Received 2 xii 68

Steroid hormones take part in the regulation of the growth and mass of the thymo lymphatic tissue Thus adrenalectomy and castration cause lymphatic hyperplasia while excess of steroids causes lymphatic atrophy (for ref see *Dougherty* 1952) The steroid induced lymphatic involution is most pronounced in the thymic cortex Nuclear pyknosis and cellular lysis occur within the first hours after administration of steroids Inhibition of the synthesis of proteins and nucleoproteins by the thymolytic steroids has also been reported (for ref see *Ernstrom & Larsson* 1967) After a delay of some days the steroids cause a marked reduction in the export of thymic lymphocytes at least in guinea pigs (*Ernstrom & Larsson* 1967)

The first question to be investigated in the present paper was the possible influence of steroids on the output of lymphocytes from the spleen either early during the phase of involution or late during the phase of lymphatic regeneration

In several rodents the thymus is necessary for normal growth and differentiation of the non thymic lymphatic tissue during early post natal stages (*Axsenberg et al* 1962 *Arnason et al* 1962 *Martinez et al* 1962 *Miller* 1962 *Sherman et al* 1963) In animals more mature at birth (e g the guinea pig) and in older animals thymectomy has less or no significant influence on the mass or histological appearance of the lymphatic organs In agreement herewith no conspicuous influence of thymectomy on the output of lymphocytes from the normal spleen in guinea pigs could be demonstrated (*Ernstrom et al* 1969)

Even in mature animals however quantitative studies have disclosed a role of the thymus in the regeneration of lymphatic tissue after involution caused by X irradiation (*Globerson et al* 1962 *Miller* 1962

The expenses of this investigation were defrayed by a grant from the Swedish *Cancer Society*

Miller et al 1963 *Cross et al* 1964 *Globerson & Feldman* 1964) In a previous paper an influence of the thymus on the regeneration of the spleen after steroid induced involution was demonstrated in guinea pigs—regeneration being retarded in thymectomized animals (*Ernstrom & Gyllensten* 1965)

The second question investigated in the present paper was the possible influence of the thymus on the output of lymphocytes from the spleen during steroid induced involution and regeneration

MATERIAL AND METHODS

Totally 200 male guinea pigs with an initial weight of 212 ± 3 g (mean ± S E.) were used The animals were fed on cabbage turnips carrots and vitamin pellets They were divided into two main groups

Sham operated guinea pigs injected with prednisolone (99 animals)

Thymectomized guinea pigs injected with prednisolone (102 animals)

Thymectomy and sham operation were performed under local anesthesia (subcutaneous infiltration of 0 5 per cent Xylocaine® Astra) according to the technique of *Gyllensten* (1953)

Prednisolone (Ultracortenol® Ciba) was administered i p in a single dose of 0 mg kg b w The injection was given immediately after operation

The animals were investigated 1 3 and 6 hours and 1 3 6 and 9 days after operation and steroid treatment On investigation the guinea pigs were anaesthetized with 2 5 per cent Nembutal sodium (25–50 mg/kg b w) Blood samples were taken from a splenic vein and a splenic artery (for details see the previous paper in this series)

The blood samples were used for white cell counts in a Bürker counting chamber with differentiation between polynuclear and mononuclear cells, and for differentiation of lymphocytes into subclasses (including cells with different mitochondrial content) in preparations stained supravitally with Janus green B and neutral red (for details see *Ernstrom et al* 1969)

Lymphocytes with 0–10 mitochondria are denoted as cells with low mitochondrial content (low MC) lymphocytes with 11 20 mitochondria as cells with medium mitochondrial content (medium MC) and lymphocytes with >20 mitochondria as cells with high mitochondrial content (high MC)

The number of lymphocytes and granulocytes per mm³ of blood from the splenic artery and the splenic vein was calculated The splenic veno arterial differences in the number of different white blood cells were obtained from each animal and all differences were then analysed statistically by Student s *t* test

RESULTS

Sham Operated Animals Treated with Steroid

The total number of lymphocytes was only slightly depressed by steroid treatment Minimal values were recorded at 6 hours and 6 days after steroid administration with a transient increase to the normal level at 1 day

A subdivision of the lymphocyte population showed that a biphasic change in the lymphocyte number in the blood after steroid injection occurred in all subpopulations although most markedly in the lymphocytes with low and medium mitochondrial content (Fig 1)

The splenic veno arterial difference in number of lymphocytes was not depressed by steroid treatment—normal difference being about 200 cells per mm³ (*Ernstrom et al* 1969) On the contrary a high

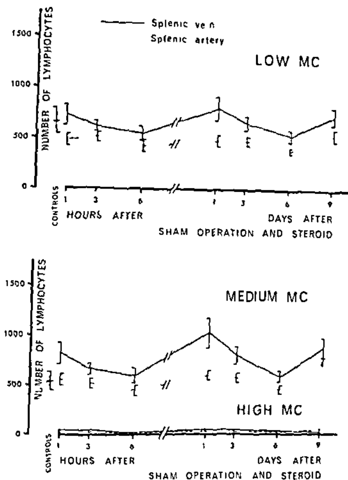


Fig 1

Number of lymphocytes per mm³ of blood from a splenic vein and a splenic artery at different intervals after sham operation and treatment with prednisolone The lymphocytes are subdivided into cells with low medium and high mitochondrial content (MC) Mean ± SE

export value was registered 1 day after steroid the difference being 772 ± 254 cells (Fig 2) A subdivision of the lymphocytes showed that all categories of lymphocytes were exported in large number at this time (Fig 3) A significant export of small lymphocytes with low mitochondrial content was found at all time intervals after treatment (Fig 3) A significant export of medium sized lymphocytes with medium mitochondrial content also occurred at all times except 9 days after treatment (Fig 3)

Pronounced granulocytosis occurred 3 and 6 hours after steroid administration At 24 hours the number of granulocytes *in the blood* was normal again (Fig 4a) Apart from this early granulocytosis no change in blood granulocytes was recorded

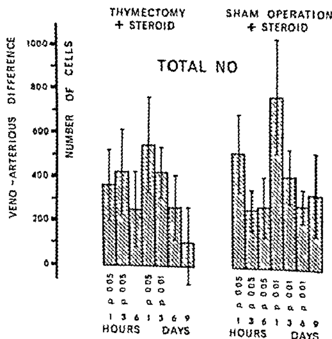


Fig 2

Difference between number of lymphocytes per mm^3 of blood from a splenic vein and a splenic artery at different intervals after sham operation or thymectomy and treatment with prednisolone Mean ± S E.

Thymectomized Animals Treated with Steroid

No significant depression of the total lymphocyte count occurred As in the sham operated animals a high lymphocyte number was noted after 1 day and lower values after 3 6 and 9 days A subdivision of the lymphocyte population demonstrated that both lymphocytes with low and medium mitochondrial content followed the same course (Fig 3)

The splenic veno arterial difference in number of lymphocytes was high 1 day after steroid injection and operation as in the sham operated animals This difference subsequently decreased successively to a minimal value of 109 ± 192 cells per mm^3 after 9 days (no signifi cant export Fig 2) The subdivision of the lymphocyte population demonstrated that the lymphocytes with low and medium mitochon drial content behaved identically i e a large difference was present after 1 day followed by a decreasing difference to a minimal value after 9 days (Fig 3) As regards the smallest type of lymphocytes with 0 5 mitochondria per cell the splenic output was lower in the thymec tomized than in the sham-operated animals at all corresponding inter vals after steroid treatment This deficient export in the thymectomized steroid treated guinea pigs was most pronounced after 9 days (Table 1)

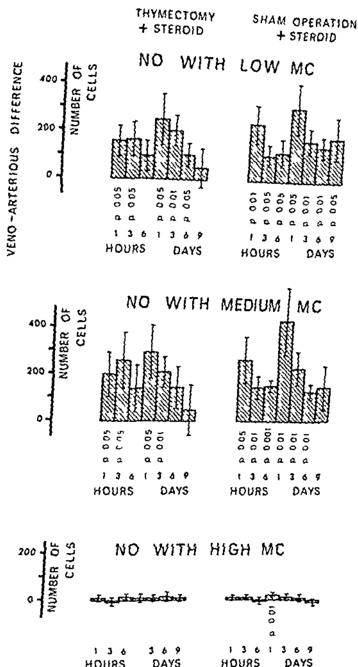


Fig 3

Difference between number of lymphocytes per mm³ of blood from a splenic vein and a splenic artery at different intervals after sham operation or thymectomy and treatment with prednisolone. The lymphocytes are subdivided into cells with low, medium and high mitochondrial content (MC). Mean ± SE

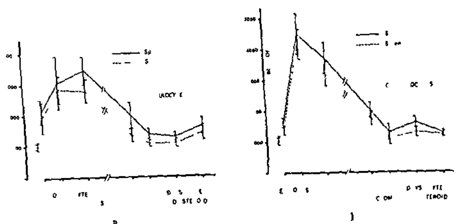


Fig 4

Number of granulocytes per mm³ of blood from a splenic vein and a splenic artery at different intervals after sham operation (a) or thymectomy (b) and treatment with prednisolone Mean ± S E

TABLE 1

Splenic Veno Arterious Difference in Number of Lymphocytes Belonging to the Class Characterized by 0–5 Mitochondria per Cell (Smallest Type of Lymphocytes)

	Interval from operation and treatment						
	1 h	3 h	6 h	1 day	3 days	6 days	9 days
Thymectomized steroid treated	22±12	12±11	14±10	23±18	9±11	8±15	0±18
Sham operated, steroid treated	28±12	17±9	15±7	40±16	22±8	10±10	28±10

Lower values in thymectomized than in sham operated animals No per mm³ Mean difference ± S E denotes significant export $p < 0.05$

The granulocytes increased in number from the normal values of around 1000 cells per mm³ to about 4000 cells at 3 and 6 hours after thymectomy and steroid administration (Fig 4b)

DISCUSSION

In guinea pigs with intact thymus steroid treatment caused no depression of the splenic export of lymphocytes This is in contrast to the decreased export from the thymus (*Ernstrom & Larsson* 1967) The difference in response to steroids indicates the existence of biological differences between thymic and splenic lymphocytes

The present investigation as well as a previous one (*Ernstrom & Larsson* 1967) disclosed a large number of circulating blood lymphocytes 24 hours after steroid treatment irrespective of thymectomy Concurrently a large export of lymphocytes from the spleen was

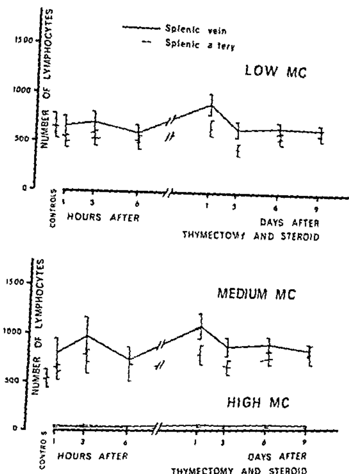


Fig 5

Number of lymphocytes per mm³ of blood from a splenic vein and a splenic artery at different intervals after thymectomy and treatment with prednisolone The lymphocytes are subdivided into cell with low medium and high mitochondrial content (MC) Mean ± SE

found the export including all categories of lymphocytes It is not known whether the emigrating splenic cells are important for the regeneration of other lymphatic tissues damaged by the steroids

In the thymectomized guinea pigs treated with steroid the splenic export of lymphocytes decreased during the phase of lymphatic regeneration (3 6 and 9 days after steroid —*Gyllensten* 1962) At 9 days after steroid administration the total export was only 30 per cent of that in the sham operated animals and no export of the smallest type of lymphocytes occurred This decrease was much more pronounced than that after thymectomy without steroid (*Ernstrom et al* 1969)

Thus thymectomy interferes with the export of lymphocytes from the spleen during lymphatic regeneration after steroid induced involution This finding may be regarded as an analogue to the retarded

regeneration of steroid involuted lymphatic tissue in thymectomized guinea pigs (*Ernstrom & Gyllensten* 1965) and to the delayed immunological restitution after irradiation of the lymphatic tissue in thymectomized adult mice (*Globerson et al* 1962 *Miller et al* 1963)

The result may be explained by a migratory stream of lymphocytes from the thymus to the regenerating spleen this flow of cells being necessary for normal splenic regeneration and export of lymphocytes. However the additional influence of a thymic hormone on the spleen cannot be excluded

SUMMARY

The lymphocyte populations of blood from a splenic vein and a splenic artery were studied in sham operated and thymectomized guinea pigs during steroid induced involution and regeneration of the lymphatic tissue Special attention was focused on the splenic veno arterial difference in lymphocyte number indicating an export or import of lymphocytes in the spleen The following observations were made

1 At 24 hours after steroid administration the blood contained a large number of lymphocytes irrespective of thymectomy Concurrently a large export of splenic lymphocytes occurred also irrespective of thymectomy

2 In sham operated guinea pigs no depression of the splenic export of lymphocytes was noted after steroid treatment

3 In the thymectomized guinea pigs the splenic export of lymphocytes decreased during lymphatic regeneration after steroid induced involution This was most pronounced in the smallest lymphocytes A migration of small lymphocytes from the thymus to the spleen may be necessary for normal splenic regeneration

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HISTOLOGICAL STAINING PROPERTIES OF *IN VITRO* FORMED FIBRIN CLOTS AND PRECIPITATED FIBRINOGEN

By

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Received 1 x 68

Common techniques for the demonstration of fibrin in histological sections are immunofluorescence technique and various histological staining methods among which Mallory s phosphotungstic acid haematoxylin (PTAH) (19) and the trichrome methods with modifications (14) are most commonly used

The immunofluorescence technique is believed to be the one most specific for the demonstration of fibrin (5 13) The PTAH and the trichrome methods seem to be more unspecific although these methods are used in histological routine work more than the others

The mechanism of the staining reactions obtained by using the PTAH and the trichrome methods is largely unknown (2 14) Occasionally the demonstration of fibrin by the PTAH or trichrome methods seems to fail Thus if fibrin clots are formed by purified fibrinogen and thrombin alone a negative result is obtained with the PTAH (8) and Lendrum s Martius scarlet blue (MSB) (10) methods This may be related to the lack of fibrin stabilizing factor (FSF) which is suggested to promote the formation of disulphide bonds resisting the action of solvents such as 30 per cent urea or 1 per cent monochloracetic acid (16) However electron microscopical examinations of fibrin prepared from normal plasma and plasma from a patient with FSF deficiency showed no structural difference between the two types of fibrin when stained with phosphotungstic acid (6 12)

The characteristics of the fibrin clot are probably influenced by several factors other than FSF Thus fibrin aggregation is enhanced by calcium (9) as well as by colloids (1)

Fibrinogen the precursor of fibrin is easily precipitated *in vitro* without actual transformation into fibrin (7 9) To what extent

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precipitation occurs *in vivo* is unknown but it may be a potential source of error in histological staining of fibrin

The aim of this study is to examine under which conditions fibrin fibrinogen and globulins treated by histological technique give the specific staining reactions when the PTAH method the trichrome methods and the immunohistochemical technique are used

MATERIALS

Fibrinogen Purified human fibrinogen (Kabi Stockholm Sweden) about 95 per cent clottable was used It was dissolved in 0 3 M NaCl adjusted to pH 7 4 with 1 N NaOH and dialysed against 0 3 M NaCl buffered with 1/10 volume Owren s buffer for 4 hours After the dialysis the concentration of fibrinogen was 1 2 per cent

Citrated platelet poor plasma Nine volumes of blood from healthy fasting subjects were collected into bottles containing one volume of 3 12 per cent sodium citrate solution After centrifugation for 60 minutes at 2000 g the plasma was pipetted off recentrifuged and stored at −20 C.

Plasma with defect FSF (Fibrin Stabilizing Factor) function Platelet poor citrated plasma was obtained from a 24 years old female patient (kindly supplied by Dr H C Godal) She had a moderate bleeding tendency (haemorrhages after partus and after tooth extraction) Laboratory examination revealed no other coagulation defect than a deficient FSF function Further studies disclosed the presence of a strong inhibitor of FSF in the patient s plasma.

Serum Whole blood from normal subjects were collected in a glass bottle and incubated at 37 C for one hour After removal of the clot by centrifugation for 30 minutes serum was incubated at 37 C overnight. The stored serum did not clot fibrinogen

Serum fibrinogen mixture Fibrinogen was dialysed against a 3 12 per cent sodium citrate solution for 24 hours at 4 C. One volume of this solution was mixed with 4 volumes of normal serum

Macroglobulin serum was obtained from a 80 years old male patient with macroglobulinaemia Waldenström The patient presented moderate anaemia typical sternal marrow findings and a moderate bleeding tendency The serum contained about 3 per cent of γM globulin

Purified macroglobulin One volume of serum from the patient with macroglobulinaemia was diluted with 9 volumes of distilled water After centrifugation the supernatant was discarded The precipitate was dissolved in 0 3 M NaCl and reprecipitated twice

Owren s buffer A modified veronal buffer pH 7 38 ionic strength 0 15 (20)

Thrombin Highly purified human thrombin was prepared by the method of Berg et al (3) and contained 30 NIH units per ml in distilled water

Human serum albumin A 20 per cent aqueous solution of human serum albumin was obtained from Kabi Stockholm Sweden This solution was diluted with distilled water to a final concentration of 4 per cent

Dextran A 10 per cent solution of Dextran 40 Rheomacrodex in 0 15 M NaCl (Pharmacia Uppsala Sweden) Dilutions were performed with 0 15 M NaCl

Glycine methyl ester HCl (Sigma Chemical Company St Louis Missouri U S A) An 0 1 M solution was adjusted to pH 7 4 Glycine methyl ester is an inhibitor of fibrin cross linking (18)

Antisera The following commercial antisera (Hyland Laboratories Los Angeles California U S A) were used

rabbit anti human fibrin labelled with fluorescein isothiocyanate FITC)
rabbit anti human fibrinogen labelled with FITC
rabbit anti human albumin labelled with FITC
goat anti rabbit γ globulin labelled with FITC

By immunoelectrophoresis against normal human plasma the antiserum against fibrin showed one precipitation line in the β region Anti fibrinogen serum showed a distinct line in the same position and a very faint line probably corresponding to α macroglobulin Anti albumin showed only one precipitation line

By gel diffusion tests anti fibrinogen serum showed one precipitation line against plasma diluted 1 2 and 1 10 and no line against normal human serum diluted 1 2 and 1 10 Anti albumin serum showed one precipitation line against human serum albumin (1 mg/ml) and normal human serum diluted 1 5 and 1 20

METHODS

Clots

The clots were prepared in lustroid tubes Unless otherwise stated the tubes were immersed in a waterbath at 37 C for three hours prior to the addition of fixation fluid

Fibrin clots prepared from purified fibrinogen The fibrinogen solution (0 2 ml) was mixed with one of the following test solutions at 37 C

(1) 0 2 ml of Owren s buffer + 0 2 ml of H_2O
(2) 0 2 ml of 50 mM $CaCl_2$ + 0.2 ml of H_2O
(3) 0 2 ml of 50 mM $CaCl_2$ + 0 2 ml of glycine methyl ester
(4) 0.2 ml of albumin + 0 2 ml of Owren s buffer

Thereafter 0 2 ml of thrombin was added One sample with each test solution was left undisturbed after addition of thrombin Clotting took place within 10 seconds Another sample was stirred with a glass or wooden rod during clotting after addition of thrombin

Other samples with test solution (1) and thrombin were incubated for three hours Thereafter ultracentrifugation was carried out (Spinco L 2 rotor 40) at 35 000 rpm (111 000 g at tip) for three hours at 35 -37 C or incubated at 2 C for three hours prior to fixation

Fibrin clots prepared from citrated plasma Citrated plasma (0 2 ml normal or with defect FSF function) was mixed with

(1) 0 4 ml of Owren s buffer
(2) 0 2 ml of 50 mM $CaCl_2$ + 0 2 ml of Owren s buffer

and 0 2 ml of thrombin was added One sample with either test solution was left undisturbed another sample was stirred Samples with test solution (1) were also subjected to ultracentrifugation as described above

Fibrin clots prepared from mixtures of serum and fibrinogen These were made and treated as clots from citrated plasma

Whole blood clots Samples of one ml of whole blood from normal subjects were collected with out anticoagulant Without stirring clotting took place in about one hour In the samples which were stirred with a glass rod clotting took place in about 30 minutes

Solubility The clots formed by addition of thrombin to purified fibrinogen were soluble in 30 per cent urea or one per cent monochloracetic acid within 3 hours and insoluble when formed in the presence of $CaCl_2$

Clots formed by addition of thrombin to purified fibrinogen in the presence of $CaCl_2$ and glycine methyl ester were soluble in urea or monochloracetic acid within 3 hours thus demonstrating inhibition of FSF Likewise clots formed by addition of thrombin to citrated plasma with defect FSF function in the presence of $CaCl_2$ were soluble in urea or monochloracetic acid within 3 hours

Precipitates

Precipitates were prepared in lustroid tubes at 2 C After incubation the tubes were centrifuged at 7000 g for 30 minutes The supernatant was removed and fixation fluid added to the precipitates

Purified fibrinogen Fibrinogen solution (0 2 ml) was diluted with
(1) 5 ml of 0 014 per cent acetic acid
(2) 0 4 ml of 50 mM $CaCl_2$ + 2 5 ml H_2O
(3) 2 ml of 15 per cent dextran
Incubation time was 3 hours

Citrated plasma

(1) 0 2 ml of citrated plasma + 4 5 ml of 0 014 per cent acetic acid
(2) 1 ml of citrated plasma + 0 5 ml of 10 per cent dextran
Incubation time was 18 hours

Serum fibrinogen mixture One ml of serum fibrinogen mixture was diluted with 0 5 ml of 10 per cent dextran Incubation time was 18 hours

Euglobulin fractions Normal serum (0 5 ml) serum from a patient with macroglobulinaemia Waldenstrom (0 5 ml) or purified macroglobulin solution (0 5 ml) was diluted with

(1) 9 5 ml of 0 014 per cent acetic acid

(2) 0 1 ml of 3 12 per cent sodium citrate + 9 5 ml of 0 014 per cent acetic acid

Incubation time was 10 minutes

Preparation and Staining of Sections

Studies using conventional histological technique All clots and precipitates were fixed in Zenker s fluid for 24 hours and rinsed in flowing tap water for another 24 hours or fixed in 8 per cent formaldehyde with 5 per cent mercuric chloride for 3 days

Specimens fixed in Zenker s fluid were stained with Mallory s phosphotungstic acid haematoxylin (PTAH) (19) Specimens fixed in formaldehyde/mercuric chloride were stained with Heidenhain s azan stain (29) Lendrum s Martius scarlet blue method and Masson 44/41 method (14)

In the following red stain using MSB and Heidenhain s azan methods dark blue using Masson 44/41 and bluish black using PTAH will be designated as + (positive) Blue stain using MSB Heidenhain s azan and Masson 44/41 methods as well as red brownish stain using PTAH method will be designated as – (negative) Where intermediate staining occurred the symbol +– will be used In some sections the material mostly gave a positive stain but in smaller areas the stain was negative or vice versa These instances will be marked +(–) or –(+) respectively

Immunofluorescence studies The following specimens were used

(1) fibrinogen precipitated from citrated plasma with acetic acid

(2) euglobulin fractions from serum

(3) euglobulin fractions from macroglobulin serum

(4) purified macroglobulin

(5) fibrin clots prepared from purified fibrinogen

(6) as (5) with addition of albumin

(7) fibrin clots prepared from citrated plasma

(8) as (7) with CaCl

The precipitates were placed in a slit in a piece of liver from mouse

The specimens were frozen by CO gas sectioned immediately at 6–8 micron in a Tissue Tek Cryostat (Ames Lab Tek) and stained the same day using the direct method of *Coons & Kaplan* (4) The sera were applied undiluted and diluted 1 1 with 0 3 M NaCl

Fluorescence microscopy was performed with a Leitz Orthomat microscope usin LG 1 and BG 38 filtres Photographs were taken with Agfa Isopan IFF film (15 din)

Controls

(1) Unstained sections showed scarcely visible autofluorescence

(2) There was no binding of goat anti rabbit γ globulin labelled with FITC

(3) Anti fibrin and anti fibrinogen labelled with FITC were adsorbed with equal amounts 0 6 per cent purified fibrinogen Anti albumin labelled with FITC was adsorbed with equal amounts of 1 per cent human serum albumin By gel diffusion tests anti fibrinogen labelled with FITC adsorbed with fibrinogen showed a precipitation line against anti fibrinogen labelled with FITC and no line against normal human plasma diluted 1 ? and 1 10 Anti albumin labelled with FITC adsorbed with albumin showed one precipitation line against anti albumin labelled with FITC The anti fibrinogen and anti albumin sera are therefore adsorbed in excess Sections treated with adsorbed sera showed no fluorescence except for the autofluorescence

RESULTS

Studies Using Conventional Histological Technique

Clots

The results of staining reactions of fibrin clots are shown in Tables 1 2 and 3

Clots from normal plasma plasma with defect FSF function and serum fibrinogen mixture stained identical

Stirring with wooden rods and stirring with glass rods gave identical staining results

TABLE 1

Fibrin Clots Formed from Purified Fibrinogen and Thrombin

	PTAH	MSB Masson 44/41 Heidenhain s azan
Purified fibrinogen	—	—
Purified fibrinogen with		
Stirring	+ (—)	+
Ultracentrifugation	+	—
Cooling	+ (—)	+
CaCl	+	+
CaCl + Stirring	+	+
CaCl + Glycine methyl ester	+	+
Albumin	+	—
Albumin + Stirring	+	+

TABLE 2

Fibrin Clots Formed from Citrated Plasma and Thrombin

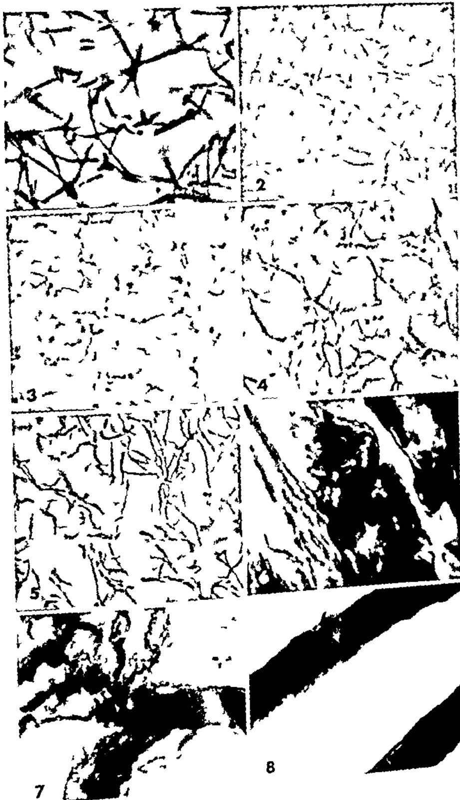
	PTAH	MSB Masson 44/41 Heidenhain s azan
Citrated plasma	+	—
Citrated plasma with		
Stirring	+	+
Ultracentrifugation	+	—
CaCl	+	—
CaCl + Stirring	+	+

Clots prepared from plasma with defect FSF function behaved like those from normal plasma.

TABLE 3

Clots Prepared from Whole Blood

	PTAH	MSB Masson 44/41 Heidenhain s azan
Whole blood	+	—
Whole blood + Stirred	+	+



PTAH. All fibrin clots derived from fibrinogen or normal plasma and formed by the addition of $CaCl_2$ revealed coarse threads (Fig. 1), whereas the lack of calcium or fibrin stabilizing factor (FSF) tended to result in more delicate fibrin strands (Figs. 2–5).

When stirring was performed during clotting the clots were composed of twisted bundles of fibres of varying thickness (Fig. 6). Clots prepared by purified fibrinogen and thrombin and kept at [illegible] C before fixation had a similar appearance (Fig. 7).

Ultracentrifugation after the clot was formed gave a central compact mass whereas delicate fibrillar structures could be discerned in the periphery (Fig. 8).

A positive stain for fibrin was dependent on the addition of $CaCl_2$ or the presence of serum colloids (whole serum or purified albumin). Thus clots formed by purified fibrinogen and thrombin gave a negative stain (Fig. 2), whereas all other clots gave a positive reaction (Figs. 1 and 3–8).

MSB. Masson 44/41. Heidenhain's azan. In fibrin clots the thickness of the individual strands varied less than the thickness of strands stained with PTAH. However, clots formed by purified fibrinogen and thrombin and by purified fibrinogen and thrombin with the addition of glycine methyl ester and $CaCl_2$ had definitely finer strands than the others.

Stirring during clotting, cooling or ultracentrifugation of the clot revealed a microscopical appearance similar to that described for PTAH stained clots.

Figs. 1–8

Fig. 1. Fibrin clot formed by citrated plasma and thrombin with the addition of $CaCl_2$. The strands are coarse. PTAH gives a positive stain. PTAH × 1200.

Fig. 2. Fibrin clot formed by purified fibrinogen and thrombin. The strands are very fine. PTAH gives a negative stain. PTAH × 1200.

Fig. 3. Fibrin clot formed by purified fibrinogen and thrombin with the addition of $CaCl_2$ and glycine methyl ester for inhibition of FSF-contaminants. The strands are very fine. PTAH gives a positive stain. PTAH × 1200.

Fig. 4. Fibrin clot formed by purified fibrinogen and thrombin with the addition of albumin. The strands are fine. PTAH gives a positive stain. PTAH × 1200.

Fig. 5. Fibrin clot formed by citrated plasma from a patient with defect FSF function and thrombin with the addition of $CaCl_2$. The strands are fine. PTAH gives a positive stain. PTAH × 1200.

Fig. 6. Fibrin clot formed by purified fibrinogen and thrombin. The sample was constantly stirred with a glass rod during clotting. The clot is composed of more or less densely packed and twisted bundles of fibrin strands. PTAH gives a positive stain. PTAH × 1200.

Fig. 7. Fibrin clot formed by purified fibrinogen and thrombin. The sample was [illegible] before fixation. The fibrin strands are packed in coarse bundles. PTAH gives a positive stain. PTAH × 1200.

Fig. 8. Fibrin clot formed by purified fibrinogen and thrombin. Ultracentrifugation was carried out after the clot was formed. The clot has a compact centre whereas delicate strands may be discerned at the periphery. PTAH gives a positive stain. PTAH × 1200.

In all sections the MSB Masson 44/41 and Heidenhain s azan stains gave corresponding results

A positive stain was dependent on the addition of CaCl or on physical alterations (stirring or cooling) of the clot The presence of serum colloids (whole serum or purified albumin) seemed to prevent fibrin from taking the positive stain even when CaCl was added However, a positive stain was always attained when stirring was carried out during clotting

Likewise whole blood gave a negative reaction (Table 3) If stirring was *performed during clotting* the reaction was again reversed

Precipitates

Fibrinogen precipitated with dextran showed traces of fibrillary structure *The other precipitates were composed of granular* homogeneous masses In the presence of CaCl the granules appeared more coarse

Except for two specimens (see Tables 4 and 5) the MSB Masson 44/41 and Heidenhain s azan gave corresponding results

Fibrinogen precipitates (Table 4) Purified fibrinogen precipitated by dilution with acetic acid or CaCl gave an overall positive staining result by all methods investigated

When purified fibrinogen was precipitated in the presence of dextran an intermediate staining *reaction occurred*

When serum colloids were present PTAH still gave a positive stain *whereas the other methods* were negative

Serum precipitates (Table 5) Euglobulin from normal serum showed intermediate staining reactions with PTAH and negative reactions with the other methods

Precipitated macroglobulin gave a positive stain with MSB Masson 44/41 and Heidenhain s azan whereas *PTAH was negative*

TABLE 4

Fibrinogen Precipitates

	PTAH	MSB Masson 44/41 Heidenhain s azan
Purified fibrinogen with		
Acetic acid	+ (−)	+ (—)
CaCl	+	+
Dextran	+ −	+ —
Citrated plasma with		
Acetic acid	+ (—)	+ −
Dextran	+	
Serum fibrinogen mixture with		
Dextran	+	−

MSB was negative Masson 44/41 and *Heidenhain s azan* were positive

TABLE 5

Serum Precipitates

	PTAH	MSB Masson 44/41 Heidenhain's azan
Euglobulin from normal serum	+ —	—
Euglobulin from macroglobulin serum	—	+
Purified macroglobulin	—	+

Identical reactions were obtained with citrated serums, except for MSB was positive Masson 44/41 and Heidenhain's azan were negative

TABLE 6

Immunofluorescence Studies

	anti fibrin	anti fibrinogen	anti albumin
Clots			
Purified fibrinogen Thrombin	+	+	—
Purified fibrinogen Albumin – Thrombin	+	+	(+)
Citrated plasma Thrombin	+	+	+
Citrated plasma CaCl Thrombin	+	+	+
Precipitates			
Citrated plasma Acetic acid	+	+	+
Euglobulin from serum	—	—	(+)
Euglobulin from macroglobulin serum	—	—	(+)
Purified macroglobulin	—	—	—

(+) is indicated where weak fluorescence was obtained

Immunofluorescence Studies (Table 6)

Fibrin clots and fibrinogen precipitates showed specific fluorescence for both fibrin and fibrinogen (Figs 9 and 10)

Fibrinogen precipitated from citrated plasma showed binding of anti albumin labelled with FITC and mostly at the surface (Fig 11) Similar results were obtained in fibrin clots formed from citrated plasma by thrombin

Euglobulin fractions precipitated from serum and macroglobulin serum showed a weak fluorescence for albumin Precipitates from purified macroglobulin showed a minimal fluorescence for albumin which could not be reproduced in photographs

Serum euglobulin fractions and purified macroglobulin showed no binding of anti fibrin or anti fibrinogen labelled with FITC

There was no binding of antisera to mouse liver



Figs 9-11

Fig 9 Fibrin clot formed by citrated plasma and thrombin Anti human fibrinogen labelled with FITC × 400

Fig 10 Fibrinogen precipitate from citrated plasma produced by acetic acid Anti human fibrin labelled with FITC × 400

Fig 11 Parallel section to that of Fig 10 Anti human albumin labelled with FITC × 400

DISCUSSION

In the present study a positive stain for fibrin was found to be dependent on the addition of CaCl no matter which method under investigation were used Furthermore a positive stain with PTAH was dependent on the presence of serum colloids whereas physical factors during clotting seemed to influence the stainability when the trichrome methods (MSB Masson 41 41 Heidenhain s azan) were used Whether or not the fibrin clot was urea soluble did not seem to affect the staining reactions

Both calcium and fibrin stabilizing factor (FSF) are required for the conversion of urea soluble fibrin to the urea insoluble type (15) When one of these substances is lacking or neutralized the fibrin remains urea soluble A reduction of the effect of FSF was attained by inhibition of the FSF contaminants in purified fibrinogen (17) by glycine methyl ester (18) or by use of plasma from a patient with a strong FSF inhibitor

Fibrin clots formed by purified fibrinogen and thrombin gave a negative stain whereas clots prepared from purified fibrinogen with added CaCl gave a positive stain whether or not glycine methyl ester was present Furthermore fibrin clots prepared by plasma from a patient with a FSF inhibitor stained like fibrin clots prepared by normal plasma Thus a positive stain for fibrin is dependent on CaCl but the presence of FSF activity does not seem to be a prerequisite for the positive reaction

However in histological sections the structure of the fibrin strands seems to be dependent on the urea solubility of the clot since all normal clots with the addition of CaCl showed coarse strands whereas the

lack of calcium of FSF tended to give finer strands *Duckert et al* (6) and *Lorand et al* (18) found no difference in the histological appearance of the fibrin clots prepared from plasma with calcium whether or not FSF was present but their experimental conditions differed from ours Since under the present experimental conditions FSF appears to be necessary for the formation of coarse fibrin strands our results may also indicate that the staining reactions are not definitely influenced by the calibre thickness of the individual strands

The presence of albumin in fibrin clots formed from purified fibrinogen resulted in a positive stain with PTAH This is in accordance with the results obtained by *Gitlin & Craig* (8) who got a positive stain with PTAH when albumin was present However these authors did not relate the positive reaction to the presence of albumin but to the urea insoluble form of fibrin an explanation which does not fit with our results

Purified fibrin clots with additional albumin and fibrin clots formed from plasma gave a negative stain with the trichrome methods even if $CaCl_2$ was added Therefore the presence of serum colloids during clotting seems to promote the positive PTAH stainability of fibrin whereas such substances apparently prevent fibrin from taking the positive stain with the other methods investigated

Aggregation of fibrin strands was produced by stirring or cooling during clotting or ultracentrifugation of the clot after it was formed All these procedures interfered with the staining reactions As to stirring or cooling the effect was particularly striking for the staining with the trichrome methods since this treatment of the clot always resulted in positive staining reactions independent of the clotting medium With constant stirring during clotting the mechanical distorsion of the clot produced alterations which cannot be related to increased aggregation of fibres only since ultracentrifugation which resulted in compression of the clots gave negative staining reactions with the three mentioned methods With regard to PTAH however fibre compression may play a role since clots formed during stirring or ultracentrifugation after the clot was formed both gave a positive stain

Like fibrin clots a positive stain of fibrinogen *in vitro* is apparently dependent on the medium in which the fibrinogen is precipitated Thus on the whole the same staining pattern as that of fibrin was obtained when $CaCl_2$ was added or serum colloids were present Thus in histological sections equivocal fibrin masses are presumably difficult to distinguish from precipitated fibrinogen when standard tinctorial techniques are used In this situation the immunofluorescence technique is not useful either since fibrin and fibrinogen are immunologically indistinguishable

Precipitated globulins gave a negative stain by PTAH whereas euglobulin from macroglobulin serum and purified macroglobulin

gave a positive stain with the trichrome methods This is in agreement with previous findings in biopsy materials from patients with macroglobulinaemia (11) Thus using the trichrome methods deposits of macroglobulins or substantial adsorption of macroglobulins to fibrin (21) may be mistaken for fibrin or fibrinogen

In conclusion, among the standard tinctorial methods investigated PTAH is found to be the one most specific for the demonstration of fibrin and fibrinogen *in vitro* but it fails when fibrin clots are formed by purified fibrinogen and thrombin only The trichrome methods give a positive stain of fibrin when the clot is changed by the influence of stirring during clotting or cooling Immunohistochemical technique seems to be the method of choice for the precise demonstration of fibrin or fibrinogen in histological sections However the latter substances cannot be distinguished by any of the methods used

SUMMARY

In order to test the specificity of the common histological stains for fibrin fibrin clots and precipitates of fibrinogen and globulins were prepared by histological technique and stained with Mallory s PTAH method or one of the trichrome methods (Lendrum s MSB and Masson 44/41 and Heidenhain s azan methods) Immunofluorescence technique was also applied to the clots and precipitates

All normal fibrin clots to which CaCl was added showed coarse strands in histological sections whereas the lack of calcium or fibrin stabilizing factor (FSF) tended to give more delicate fibrin strands

A positive stain for fibrin using the PTAH method was dependent on the addition of CaCl or the presence of serum colloids

A positive stain for fibrin using the trichrome methods was dependent on the addition of CaCl or physical alterations of the clot The presence of serum colloids seemed to prevent fibrin from taking the positive stain with the trichrome methods but a positive stain was always attained when stirring was carried out during clotting

FSF is probably not necessary for a positive stain for fibrin

Fibrinogen precipitates gave also positive stain with PTAH but again this was dependent on the addition of CaCl or the presence of serum colloids With the trichrome methods fibrinogen gave a positive reaction when CaCl was added whereas the presence of serum colloids gave on the whole negative results

Euglobulin fractions of macroglobulin serum and purified macroglobulin stained mainly as fibrin when the trichrome methods were used whereas PTAH was negative

Immunofluorescence technique applied to fibrin clots and fibrinogen precipitates revealed specific fluorescence for both fibrin and fibrinogen

It is concluded that among the standard tinctorial methods investi

gated PTAH is the one most specific for the demonstration of fibrin and fibrinogen *in vitro*

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DEPOSITS OF FIBRIN AND PLASMA PROTEINS IN THE NORMAL HUMAN PLACENTA

An Immunofluorescence Study

By

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Received 1 x 68

In the normal human placenta eosinophilic extracellular *deposits of* fibrillar or granular appearance are seen in relation to the various elements of the trophoblast (Fig 1).

On the surface of the syncytium of the villi fibrin and fibrin like masses of various size are laid down (16). On the chorion plate similar deposits extend to finally form a continuous layer against the maternal blood space (Langhans layer) (16). Further masses of eosinophilic material are seen between the cells of the cell islands (14) and cyto trophoblastic cell columns (14) and of the cytotrophoblastic shell (13). In relation to the basal plate nearly continuous deposits are found both towards the intervillous space (Rohr's stria) and at the junctional zone between the cytotrophoblastic shell and the decidua (Nitabuch's layer) (13).

Many authors have studied these deposits the exact nature and origin of which have been much disputed (for review see *Moe* (13) *Moe* (14) and *Moe & Jorgensen* (16)). The major problem has been whether the deposits are composed of fibrin or other substances. Previous studies have shown that Rohr's stria and the early *deposits* on the syncytium consist of maternal platelet thrombi and that later on increasing amounts of fibrin are added (13 16). In the advanced stages the deposits on the syncytium are homogeneous by light micro scopy. Ultrastructurally they are composed of a finely granular material of moderate electron density (16). Because a few remaining platelets and scattered tracts of fibrillary fibrin were found in the granular masses it seems likely that the masses represent *old thrombi* (16). Granular masses with a similar electron microscopical appear

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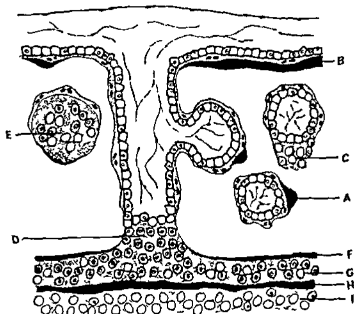


Fig 1

Deposits in the normal human placenta A) Deposits on the syncytium of the villi B) Langhans layer C) Intercellular deposits in the cytotrophoblastic cell columns D) Intercellular deposits in the anchoring villi F) Intercellular deposits in the cell islands F) Rohr's stria G) Intercellular deposits in the cytotrophoblastic shell H) Nitabuch's layer I) Decidua

ance are found in Nitabuch's layer (13) and in the intercellular deposits of the cell islands cell columns and shell (13 14) This does not necessarily mean that the composition of the granular deposit in all those sites is identical In all locations however the granular masses are mixed with varying amounts of fibrillar material with the appearance of fibrin

In the present study immunohistochemical methods were applied to placentas at different stages of pregnancy in order to obtain further information about the nature of the various deposits The presence or absence of fibrin and plasma proteins was demonstrated by applying antisera against human fibrin fibrinogen albumin and γ globulin

MATERIALS AND METHODS

Tissue specimens Five mature and 5 immature placentas from normal pregnancies were examined The immature placentas were obtained from physically healthy women whose pregnancies were interrupted on psychiatric indications after 8 to 24 weeks gestation (menstrual age) Immediately after removal the placentas were gently rinsed in 0 9 per cent NaCl solution and cut in slices Pieces for study were taken from the central and peripheral parts of the placentas Some pieces were frozen in a dry ice/ethyl alcohol mixture at —70 C and then stored at —20 C Other specimens were placed directly in the freezer at —20 C, and some pieces were frozen by CO_2 gas on a freezing stage for immediate sectioning All preparative procedures gave similar results

For comparison a *fresh* endocardial thrombus from a patient who died from myocardial infarction was frozen by CO gas and treated in the same way as the placentas

Sectioning The specimens were sectioned at 6–8 *microns* in a Tis ue Tek Cryostat (Ames Lab Tek) Alternate sections were prepared for immunofluorescence study and for light microscopy

Conventional histological techniques Sections were stained with

(1) Haematoxylin azo phloxine (HP)
(2) Lendrum s Martius scarlet blue method (MSB)
(3) Mallory s phosphotungstic acid haematoxylin method (PTAH)

For the MSB staining sections were fixed in ether/ethyl alcohol (1 1) for approximately one hour hydrated through alcohols to water postfixed in 8 per cent formaldehyde with 5 per cent mercuric chloride for 3 days and stained according to *Lendrum et al* (9) For the PTAH staining sections were fixed in ether/ethyl alcohol for approximately one hour hydrated through alcohols to water postfixed in Zenker s fluid over night and stained with the technique of *Mallory* (11)

Immunofluorescence Technique

a) *Antisera* The following commercial antisera (Hyland Laboratories Los Angeles California U S A) were used

rabbit anti human fibrin
rabbit anti human fibrin labelled with fluorescein isothiocyanate (FITC)
rabbit anti human fibrinogen
rabbit anti human fibrinogen labelled with FITC
rabbit anti human albumin
rabbit anti human albumin labelled with FITC
rabbit anti human γG globulin labelled with FITC
goat anti rabbit γ globulin labelled with FITC

By immunoelectrophoresis against normal human plasma antisera against fibrin showed one precipitation line in the β region Anti fibrinogen serum showed a distinct line in the same position and a very faint line probably corresponding to α macroglobulin Anti albumin showed only one precipitation line Anti γG globulin showed a distinct line corresponding to γG globulin and a faint one to γA globulin

By gel diffusion tests anti fibrinogen serum showed one precipitation line against plasma diluted 1 2 and 1 10 and no line against normal human serum diluted 1 2 and 1 10 Anti albumin serum showed one precipitation line against human serum albumin (1 mg/ml) and normal human serum diluted 1 5 and 1 20

Anti γG globulin serum showed precipitation lines against γG globulin (1 mg/ml) γM globulin (1 mg/ml) and pepsin split γG (1 mg/ml) this indicated that the antiserum contained antibodies to γG globulin as well as to other γ globulins The anti γG serum will therefore be designated as anti γ globulin

The amount of specific antibody and the degree of conjugation was not determined for the different antisera Therefore when the r ctions of the different antisera are compared the degree of fluores ence is not proportional to the concentration of the corresponding antigen Significant differences in protein c n centration was indicated however when a single section showed varying intensity of fluorescence in different areas

b) *Staining technique* After sectioning staining was performed on the same day using either the direct method (9) or in some sections the "sandwich method (21) The sera were applied undiluted and diluted 1 10 with 0 9 per cent NaCl

c) *Microscopy* Fluorescence micr scopy was performed with a Leitz Orthomat microscope using UG 1 and BG 38 filtres Photographs were taken with Agfa Isopan IFF film (15 din)

Control Experiments

1 *Autofluorescence* Unstained sections showed s arcely visible autofluorescence of a grey greenish colour different from the light apple green fluorescence of FITC

2 *Non specific staining* There was n binding of goat anti rabbit γ globulin labelled with FITC

3 *Adsorption* Anti fibrin and anti fibrinogen labelled with FITC were adsorbed with equal amounts 0 6 per cent purified fibrinogen (Kabi Stockholm Sweden)

Anti albumin labelled with FITC was adsorbed with equal amounts of 1 per cent human serum albumin (Kabi Stockholm Sweden) By gel diffusion tests anti fibrinogen labelled with FITC adsorbed with fibrinogen showed a precipitation line against anti fibrinogen labelled with FITC and no line against normal human plasma diluted 1 9 and 1 10 Anti albumin labelled with FITC adsorbed with albumin showed one precipitation line against anti albumin labelled with FITC The anti fibrinogen and anti albumin sera were therefore adsorbed in excess Sections treated with adsorbed sera showed no fluorescence except for the auto fluorescence

4 *Staining of fibrin and fibrinogen* Fibrin clots were prepared from purified fibrinogen Fibrinogen precipitates were made from citrated plasma Both preparations were stained with anti fibrin and anti fibrinogen labelled with FITC. A detailed description of these experiments have been given in another paper (1a) Cross reactions for fibrin and fibrinogen were obtained for both Fibrin and fibrinogen can therefore not be differentiated with these methods and the term fibrin/fibrinogen will be used in the following text Fibrinogen precipitated from citrated plasma showed binding of anti albumin labelled with FITC, and mostly at the surface Fibrin clots formed from purified fibrinogen showed no binding of anti albumin labelled with FITC

RESULTS

The direct method and the sandwich method gave the same results in the localization of the fluorescence although more strong with the sandwich method

The fluorescence with both undiluted and diluted (1 10) antisera appeared in the same areas

In the following binding of anti fibrin and anti fibrinogen labelled with FITC will be designated as "binding of anti fibrin/fibrinogen Red stain with the MSB method and bluish black stain with the PTAH method are typical reactions of fibrin and are designated as positive

Binding of anti fibrin/fibrinogen was seen throughout the deposits with a distribution similar to that of the red colour seen in haematoxylin azo phloxine preparations Generally the deposits gave a positive reaction with the MSB and PTAH methods but within more restricted areas PTAH tended to give a positive stain to the fibrillar component of the deposits only whereas MSB gave a positive reaction to some of the homogeneous masses as well Compared with the results obtained in paraffin sections both the MSB and PTAH methods applied to frozen sections tended to give positive stain in less extensive areas (13 14 16)

Binding of Anti Fibrin/Fibrinogen

In the endocardial thrombus binding of anti fibrin/fibrinogen was seen throughout all layers but was particularly prominent at the surface (Fig 2) and at the base towards the endocardium In the MSB and PTAH preparations no obvious differences in the extent of positive stain was seen and both were similar to the distribution of antibody binding

For comparison a fresh endocardial thrombus from a patient who died from myocardial infarction was frozen by CO gas and treated in the same way as the placentas

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goat anti rabbit γ globulin labelled with FITC

By immunoelectrophoresis against normal human plasma antisera against fibrin showed one precipitation line in the β region Anti fibrinogen serum showed a distinct line in the same position and a very faint line probably corresponding to α macroglobulin Anti albumin showed only one precipitation line Anti γG globulin showed a distinct line corresponding to γG globulin and a faint one to γA globulin

By gel diffusion tests anti fibrinogen serum showed one precipitation line against plasma diluted 1 2 and 1 10 and no line against normal human serum diluted 1 2 and 1 10 Anti albumin serum showed one precipitation line against human serum albumin (1 mg/ml) and normal human serum diluted 1 5 and 1 20

Anti γG globulin serum showed precipitation lines against γG globulin (1 mg/ml) γM globulin (1 mg/ml) and pepsin split γG (1 mg/ml) this indicated that the antiserum contained antibodies to γG globulin as well as to other γ globulins The anti γG serum will therefore be designated as anti γ globulin

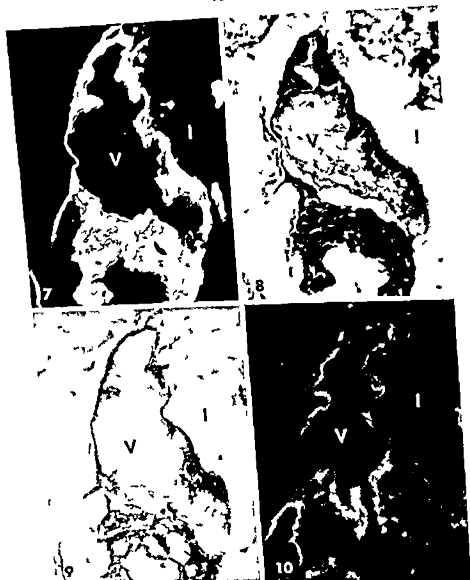
The amount of specific antibody and the degree of conjugation was not determined for the different antisera Therefore when the reactions of the different antisera are compared the degree of fluorescence is not proportional to the concentration of the corresponding antigen Significant differences in protein concentration was indicated however when a single section showed varying intensity of fluorescence in different areas

b) *Staining technique* After sectioning staining was performed on the same day using either the direct method (?) or in some sections the sandwich method (?1) The sera were applied undiluted and diluted 1 10 with 0 9 per cent NaCl

c) *Microscopy* Fluorescence microscopy was performed with a Leitz Orthomat microscope using UG 1 and BG 38 filtres Photographs were taken with Agfa Isopan IFF film (15 din)

Control Experiments

1 *Autofluorescence* Unstained sections showed scarcely visible autofluorescence of a grey greenish colour different from the light apple green fluorescence of FITC



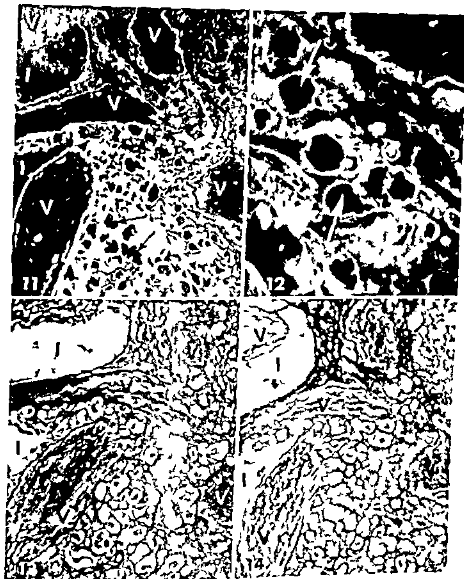
Figs 7–10

Fig 7 Placenta at 12 weeks Anti human fibrin labelled with FITC. An extensive deposit surrounding a stem villus (V) exhibits specific fluorescence Intervillous space I × 100

Fig 8 Placenta at 1 weeks Parallel section of that shown in Fig 7 Positive stain appears black Villus V Intervillous space I MSB × 100

Fig 9 Placenta at 12 weeks Parallel section of that shown in Fig 8 Positive stain appears black PTAH × 100

Fig 10 Placenta at 12 weeks Anti human albumin labelled with FITC Parallel section of that shown in Fig 9 Specific fluorescence at the periphery of the deposit against the intervillous space (I) and against the villus (V) × 100



Figs 11–14

Fig 11 Placenta at term Anti human fibrin labelled with FITC The sub horionic continuous mass (Langhan layer) exhibit specific flu rescence which is most pronounced at the periphery of the masses and a a halo around pro liferating cytotrophobla t cells which present themselves as small dark defects (arrows) Larger defects are areas f fibrous villi (V) and intervillous space (I) × 100

Fig 12 Placenta at term Anti human fibrin labelled with FITC Higher magnifica tion of Fig 11 Rounded lark d fects in areas of proliferating cyto trophoblastic cells (arrows) × 400

Fig 13 Placenta at term Parallel ection of that shown in Fig 11 Positive stain appears darl grey Collagen fibres black Fibrous villi V Intervillous space I MSB × 100

Fig 14 Placenta at term Parallel ection of that shown in Fig 13 I itive stain of fibrillar material appearing black Fibrous villi V Intervill us space I PTAH × 100

and PTAH although the stained material was not uniformly distributed (Figs 5 and 6)

Particularly in the mature placenta larger deposits were observed on the villi and the chorion plate (Figs 7 and 11) A narrow zone along the periphery gave a brighter fluorescence of anti fibrin/fibrinogen than the central parts (Fig 11) Where proliferating cytotrophoblastic cells were found within the deposits, a halo of more pronounced fluorescence was often seen around the cells (Fig 12) MSB resulted in a positive stain in great areas of the larger deposits (Figs 8 and 13) whereas PTAH gave a positive reaction in patches and streaks (Figs 9 and 14)

Both in Rohr s stria and in Nitabuch s layer a bright anti fibrin/fibrinogen fluorescence was produced throughout (Figs 15 and 18) Further in MSB and PTAH preparations abundant positive stain was seen in both layers in certain instances the positive stain was nearly as extensive as the fluorescence in the antibody preparations (Figs 17 and 20)

In the cell islands cytotrophoblastic cell columns and shell the binding of anti fibrin/fibrinogen was found intercellulary at all gestational stages (Fig 21) However the fluorescence particularly of the shell seemed to be more intensive in the mature than in the immature placentas when compared to the fluorescence of the deposits on the villi in the same section In most areas of the islands columns and shell the fluorescence was more pronounced near the surface of the deposits towards the intervillous space than in the inner parts As in the larger deposits of the villi and in Langhans layer a halo of intense fluorescence was surrounding the cells (Fig 21) Fluorescent material occurred only rarely in the cytoplasm of these cells MSB and PTAH gave a positive stain in some regions of the intercellular material of the cell islands cytotrophoblastic cell columns and shell but was negative in other areas In the cell columns and cell islands the pattern of MSB staining was in accordance with the binding of anti fibrin/fibrinogen or positive stain near the intervillous space and only scattered areas of positive stain in the central regions (Fig 22) The PTAH method showed a similar distribution of the positive stain but was in most cases completely negative in central areas (Fig 23) In the cytotrophoblastic shell both MSB and PTAH were usually negative

In the decidua only a very faint anti fibrin/fibrinogen fluorescence was present between the cells MSB and PTAH were negative

Binding of Anti Albumin

In the endocardial thrombus anti albumin (Fig 3) fluorescence was only found on the surface and was not present in the inner parts

A similar distribution was seen in the deposits on the syncytium of the villi and the chorion plate Anti albumin fluorescence was seen in

a narrow zone along the periphery of the masses both towards the maternal blood space and at the inner border against the villus or chorion plate but was occasionally present within the deposits as in the case shown in Fig. 10

In Rohr's stria anti albumin gave only a faint fluorescence (Fig. 16) as compared with that along the surface of the deposits on the villi. In Nitabuch's layer only a weak fluorescence was occasionally seen (Fig. 19)

In the cell islands cell columns and shell anti albumin gave a very



faint fluorescence in the intercellular matrix occasionally patchy areas with a brighter fluorescence were seen (Fig 24)

Intercellularly in the decidua only a faint anti albumin fluorescence was seen

Binding of Anti γ Globulin

The binding of anti γ globulin labelled with FITC nearly always showed the same localization as anti albumin labelled with FITC

In Nitabuch s layer anti γ globulin fluorescence was not present

DISCUSSION

The immunohistochemical techniques are sensitive and selective methods for the demonstration of plasma proteins in tissue sections

Many standard methods have been worked out for the staining of fibrin It is a common observation however that the immunohisto chemical methods give a more extensive distribution of positive fibrin stain than the standard tinctorial methods (3 6 8 22) This may partly be due to cross reactions between fibrin and anti fibrinogen and be tween fibrinogen and anti fibrin (15 20) Furthermore it is known that anti fibrin and anti fibrinogen react not only with fibrin and fibrinogen but also with some of the split products and degradation products of these proteins (12 18) Therefore the immunohisto

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Figs 15–20

Fig 15 Placenta at 24 weeks Anti human fibrin labelled with FITC. Rohr s stria (R) exhibits specific fluorescence *Intervillous* space I Cytotrophoblastic shell C × 400

Fig 16 Placenta at 24 weeks Anti human albumin labelled with FITC. Parallel section of that shown in *Fig* 15 *Faint* fluorescence as a halo around the cytotrophoblastic cells (arrows) Rohr s stria R Intervillous space I Cytotrophoblastic shell C × 400

Fig 17 Placenta at 24 weeks Parallel section of that shown in Fig 16 Positive stain of the deposit at the surface (arrows) At upper part a villus (V) Intervillous space I Cytotrophoblastic shell C. MSB × 400

Fig 18 Placenta at 24 weeks Anti human fibrin labelled with FITC Nitabuch s layer exhibits specific fluorescence Cytotrophoblastic shell C. Decidua D × 400

Fig 19 Placenta at 24 weeks Anti *human* albumin labelled with FITC. Parallel section of that shown in Fig 18 Area of Nitabuch s layer N Cytotrophoblastic shell C. Decidua D × 400

Fig 20 Placenta at 24 weeks Parallel section of that shown in Fig 19 Positive stain of Nitabuch s layer appears black. Cytotrophoblastic shell C. Decidua D PTAH × 400



Figs 21–2

Fig 21 Placenta at term Anti human fibrin labelled with *FITC* Fluorescence in the intercellular material of a cell island Rounded dark defects in areas of the cells (arrows) Intervillous space I × 100

Fig 22 Placenta at term Parallel section of that shown in Fig 21 Positive stain of the intercellular material peripherally and small patches centrally (arrows) Intervillous space I MSB × 100

Fig 23 Placenta at term Parallel section of that shown in Fig 22 Positive stain appear black at the periphery of the cell island PTAH × 100

Fig 24 Placenta at term Anti human albumin labelled with FITC Parallel section of that shown in Fig 23 Small patches with bright fluorescence intercellulary Intervillous space I × 100

chemical techniques demonstrate fibrin *fibrinogen* and some of their derivatives whereas conventional histological techniques stain fibrin (5 15) and fibrinogen (15) only under certain conditions as shown in *in vitro* experiments

Furthermore the immunohistochemical method is more specific

because standard tinctorial methods may give a positive stain of substances not related to fibrin e g a positive stain of γM globulin by MSB (15)

The small deposits on the syncytium gave a bright fluorescence with the anti fibrin/fibrinogen preparations

In light and electron microscopical studies of human placentas these early small deposits in relation to the villi on the chorion plate (16) and in Rohr s stria (13) were found to have the structure of platelet thrombi at various stages of transformation to fibrin thrombi The present evidence of the localization of fibrin/fibrinogen in these areas is in agreement with the previous results

Judged by the findings in the present study the large intervillous deposits particularly found in the mature placentas contained a considerable amount of fibrin fibrinogen and/or their derivatives This fits with the theory that the deposits are composed of aged fibrin assumed to be remnants of old platelet fibrin thrombi (16)

When stained with anti fibrin/fibrinogen the brighter fluorescence along the edges of these masses need not reflect any differences in the concentration of fibrin as *in vitro* immunohistochemical studies of *fibrin clots* revealed a similar staining pattern of the fibrin strands (6) However the concentration at the surface might be due to non specific reactions but the negative stain with the antiserum adsorbed in excess excludes this theory

Occasionally fibrin/fibrinogen was observed within the cytoplasm of the trophoblastic cells underlying the intervillous masses This may well be a sign of cell injury (7) and it is in accordance with the electron microscopical finding of intracellular fibrin strands at these sites (16)

Unlike the deposits bordering the intervillous space Nitabuch s layer and the intercellular materials of the cell islands cell columns and shell are not of thrombogenic origin as platelets were not found in these locations (13 14 23)

In Nitabuch s layer a constant and strong fluorescence for fibrin/fibrinogen suggests that substantial amounts of fibrin fibrinogen and/or their derivatives are present This is in good agreement with the positive MSB and PTAH stain and previous findings using other conventional techniques (10 17 19) By electron microscopy fibrils indicative of fibrin are found intermingled with granular masses and cell debris (13)

Deposition of fibrin/fibrinogen was found throughout the intercellular deposits of the cell islands cell columns and shell Although scattered fibrin fibrils were seen ultrastructurally in the intercellular deposits of these cell collections the major components were granular masses and cell debris (13 14) The binding of anti fibrin/fibrinogen to areas mostly corresponding to granular masses ultrastructurally is therefore not to any great extent due to the presence of newly formed

fibrin but is probably related either to fibrinogen or aged fibrin (16), *i e* a material similar to that of the larger thrombogenic deposits bordering the intervillous space

Anti albumin gave fluorescence only at the periphery of the intervillous deposits and Langhans layer This is contrary *to Brosko et al* (1) who found albumin throughout the intervillous deposits In the *present study* however, albumin was also located only at the periphery of the endocardial thrombus The deposition of *albumin therefore* seems to be a non specific adsorption to the surface of thrombi That *the fluorescence* is contingent upon a specific immunological reaction was shown by a negative stain with the antiserum adsorbed *in excess*

In the intercellular deposits of the cell islands cell columns and *shell* anti albumin gave a very faint fluorescence and occasionally patchy areas with a brighter fluorescence This was *in contrast to the* bright and even fluorescence of fibrin/fibrinogen throughout the deposits This could mean that fibrinogen or its derivatives constitute a component of the deposits more important than albumin At no stage of pregnancy does the placenta synthesize albumin or γ globulin (4) but the possibility exists that these cells may synthesize fibrinogen as they *exhibit secretory activity ultrastructurally* (13 14) *However it* is more likely that the fibrinogen and albumin in these locations originate from the maternal plasma leaking from the intervillous space The fact that fibrinogen *or its derivatives* were more extensively distributed than albumin may be explained by the particularly easy precipitation of fibrinogen or by formation of fibrin

Anti γ *globulin fluorescence* nearly always showed the same localization as anti albumin fluorescence The controls as regards the anti γ globulin *serum* are considered to be insufficient Staining with anti γ *globulin* labelled with FITC *may* therefore be contingent upon non specific reactions However areas *not* stained with anti γ globulin labelled with FITC *e g* Nitabuch s layer most likely do not contain substantial *amounts of* γ globulin The lack of immunoglobulins in Nitabuch s layer probably indicates that this zone does not contain immunological reaction products

SUMMARY

The deposits in the normal human placenta at various stages of gestation were studied using FITC labelled rabbit antisera *against human* fibrin fibrinogen albumin and γ *globulin*

Fibrin or fibrinogen was found to be present throughout the deposits on the syncytium of the villi and the chorion plate in Rohr s stria and in Nitabuch s layer Fibrin or *fibrinogen* was also present in the intercellular material of the cell islands and cytotrophoblastic *cell columns* at all stages of pregnancy and in the *intercellular material of the cytotrophoblastic* shell particularly at an advanced *gestational age*

Albumin was found to be present along the periphery of the deposits on the syncytium In the intercellular deposits of the cell islands cell columns and shell FITC labelled anti albumin gave a very faint fluorescence and occasionally patchy areas with a brighter fluorescence Only traces of albumin was present in Rohr s stria and Nitabuch s layer

γ globulins were not present in Nitabuch s layer

The findings are in agreement with previous evidence indicating that deposits on the syncytium and Rohr s stria develop from platelet fibrin thrombi The intercellular deposits of the cell islands cell columns, shell and Nitabuch s layer are possibly fibrin or fibrinogen precipitated from maternal plasma seeping through the tissue from the intervillous space

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SEX DIFFERENCE IN THE GROWTH OF A STRAIN SPECIFIC MOUSE TUMOUR, TA3

By

F Hartveit

Received 1 xi 68

Sex differences in the growth of transplantable tumours are said to be rare (see *Snell* 1953) but have recently been encountered at this Institute Thus the Ehrlich ascites carcinoma a non specific mouse tumour grows better subcutaneously in male mice (*Hartveit* 1962 b) in which the survival time is also less than in females (*Hartveit* 1962 a) and the strain specific TA3 carcinoma grows better in strain A males than females (*Thunold* 1966)

The following experiment again demonstrates this sex-difference in the growth of TA3 in A mice and also in their F_1 hybrids and further relates this finding to the survival time of the animals

One subcutaneous injection of 0 25 ml of a saline suspension of TA3 cells made up from a solid subcutaneous transplant in a male A mouse was given to each of 6 male and 6 female A mice and to a similar number of F_1 hybrids (strain A male crossed with females of our closed colony) The sum of the two greatest tumour diameters was measured during growth and the survival time of the mice recorded

TABLE 1
Tumour Size and Survival Time in A and F_1 Hybrid Mice with TA3 Carcinoma

Sex	Tumour size (mm) ± SD		Survival time (days) ± SD	
	A mice	F_1 mice	A mice	F mice
Male	62 5 ± 9 4	57 0 ± 1[illegible] 6	48 [illegible] ± 3 4	50 0 ± 4 3
Female	37 0 ± 11 7	[illegible]5 8 ± 14 9	59 4 ± 3 9	75 8 ± 1 8

See text.

The tumour measurements at 24 days (*i e* before the first mouse died) are shown in the Table 1 The tumour grew better in male than female

Research Fellow Norwegian Cancer Society
I am indebted to Professor *G Klein* for supplying our original transplant of the TA3 carcinoma

mice of both types (0.01>P>0.001). The difference between A and F_1 mice of the same sex was not significant. The survival time is also shown in the table. The male mice died earlier than the females of both types (A: 0.01>P>0.001; F_1: 0.001>P). The difference in the survival time of the A and F_1 male mice was not significant, but the female F_1 mice outlived the A females (0.001>P).

The present findings thus confirm that *TA3 grows better in male than in female A mice*, and extends this observation to *their F_1 hybrids*. The possibility that this sex difference in tumour growth is due to a direct *Eichwald-Silmser* effect (1955) is ruled out as TA3 arose in a female mouse, and the more remote possibility of its being due to an Eichwald-Silmser like effect due to the presence of male host cells injected with the tumour cells is excluded by Thunold's experiment in which the tumour was transplanted from a female mouse. The sex difference is therefore likely to be due to a difference in host response to the tumour cells themselves.

The findings that *tumour growth is similar in A and F_1 mice*, and that *the survival time of the F_1 mice was either similar to or greater than that of the A mice* are at variance with the general impression that a tumour may grow better in F_1 hybrids of its strain of origin (*Little* 1956). It is argued that the increased vitality of such hybrids provides a more favourable environment for tumour growth. In the present case the argument that hybrid vigour may be accompanied by a more intense immune response leading to inhibition of tumour growth would seem more pertinent as the effect was seen in female mice which may show a more intense immune response than males (*Halpern* 1964, *Hartveit* 1965). This latter explanation presupposes that TA3 carries with it some antigenic factor, e.g. tumour specific antigen or virus, that enables the mice to react against the otherwise isologous tumour cells.

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MORPHOLOGIC EFFECTS OF ETHIONINE ON THE PANCREAS OF THE CHINESE HAMSTER

A Light and Electron Microscopic Study of Degenerative Changes

By

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Received 25.x.68

Ethionine is known to produce destruction of the acinar pancreatic parenchyma (*cf. Farber* 1959, *Seifert & Gieseking* 1961, *Ekholm et al.* 1962, *Herman & Fitzgerald* 1962, *House et al.* 1963) and to induce fatty liver (*cf. Farber* 1967) and hepatic tumours (*Popper et al.* 1953). This compound may also evoke morphologic alterations in salivary (*Loring & Hartley* 1955, *Ulmansky & Ungar* 1967) and lacrimal glands (*Benson* 1964), gastrointestinal tract (*Loring & Hartley* 1955, *Kaufman et al.* 1962), testis (*Goldberg et al.* 1959) and kidneys (*Alvizouri & Warren* 1954). The damages induced by ethionine in the pancreas bear resemblance to those seen during protein deprivation (*Weisblum et al.* 1962). In animals treated with ethionine the pancreatic islets are usually said to be preserved and even to show some hyperplasia in later stages (*Farber* 1959).

The present study was initiated as an extension of our previous work on various kinds of degenerative and regenerative islet changes in the Chinese hamster (*Boquist* 1968a, b, c and 1969a). Its main purpose was to examine whether agents known to damage the acinar pancreatic parenchyma also could affect the islet cells. To the best of our knowledge there are no previous reports on the morphologic effects of ethionine in the Chinese hamster. The present investigation is confined to the degenerative pancreatic alterations occurring in this species during the first 2 weeks after the administration of ethionine. A report on the ensuing regenerative changes will be published separately (*Boquist* 1969b).

MATERIAL AND METHODS

Fourty two non diabetic adult Chinese hamsters of both sexes from 3 months to about . years of age were used. They were kept on a standard laboratory diet and water *ad libitum* and were housed in individual cages in animal rooms at a temper

This work was supported by grants from the Swedish Medical Research Council (Project No. B69 12X-718-04A).

ature of 20–22 C Twelve of these animals served as controls and received daily intraperitoneal injections of 1 ml of saline The other 30 hamsters got daily injections of DL ethionine (Sigma Chemical Company St Louis USA) that was prepared daily by dissolving the amount to be used in saline heated to 50 C The ethionine was administered intraperitoneally at a dose of 0 5 g/kg body weight Two control and five ethionine treated hamsters were sacrificed at 1 2 4 7 10 and 14 days after the beginning of the experiments There were no animals dying spontaneously At sacrifice specimens were taken from the pancreas for light and electron microscopic examination

For light microscopy pancreatic slices were fixed in 10 per cent formalin or Bouin s fixative The following stains were used van Gieson stain haematoxylin eosin aldehyde fuchsin in the modification by Maske (1955) chrome alum haematoxylin counterstained with ponceau fuchsin silver impregnation according to *Hellerstrom & Hellman (1960)* and periodic acid Schiff stain

For electron microscopy multiple pancreatic blocks of about 1 mm³ were fixed in 1 per cent osmium tetroxide in 0 34 M veronal acetate buffer adjusted to pH 7 4 The fixation was carried out at 0–4 C for 2 hours After fixation the slices were rinsed in physiological saline dehydrated in rising concentrations of ethanol and embedded in Epon 812 (*Luft 1961*) In order to find appropriate areas for the thin sections thick (1 μ) sections were stained with toluidine blue and studied in a light microscope The sections were cut on an LKB Ultrotome III and were stained with uranyl acetate and lead citrate Examination of the sections were performed in a Siemens Elmiskop I A and a Zeiss EM 9 (at 60–80 kV) and electron micrographs were taken at original magnifications of 2 000–30 000

RESULTS

Light Microscopy

Control Animals

The morphology of the exocrine pancreas appeared to conform to that of other mammals and there were no degenerative or regenerative alterations (Fig 1) The islets were normal

Ethionine Treated Animals

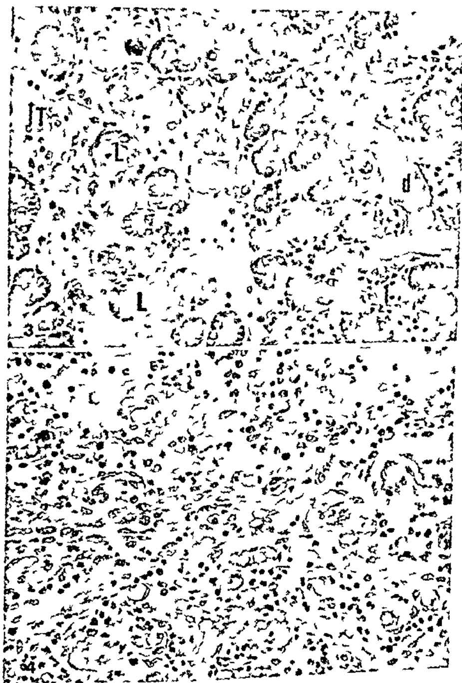
From the second day there were changes of the pancreas in all the animals These changes varied however in different parts of the same pancreas and areas with marked lesions and those with normal appearance occurred close to each other There were also variations between animals treated alike No sex or age differences were found

After 24 hours ethionine exposure scattered inflammatory cells were found in the pancreas of some animals At 2 days the acinar cells showed decrease of basal basophilia There was diffuse infiltration of polymorphonuclear leucocytes plasma cells and lymphocytes From the 4th day there were nuclear pyknosis and cytoplasmic vacuolization (Fig 2)

Figs 1 2

Fig 1 Pancreas of control hamster showing acini with small lumina The connective tissue component is inconspicuous Aldehyde fuchsin stain × 400

Fig 2 Pancreas from Chinese hamster treated with ethionine during 4 days demonstrating decreased staining affinity of the acinar cells (A) There are cytoplasmic vacuoles (v) ductules (d) and some lymphocytes Aldehyde fuchsin stain × 400



Figs 3–4

Fig 3 Pancreas from hamster treated with ethionine during 7 days showing irregular and dilated acinar lumina (L) inflammatory cells (I) increase of interstitial tissue (IT) and longitudinal section of ductule (d) van Gieson's stain × 250

Fig 4 Atrophied acinar tissue of Chinese hamster after 10 days of ethionine exposure showing polymorphonuclear leucocytes lymphocytes vacuolized cells and interstitial oedema van Gieson's stain × 250

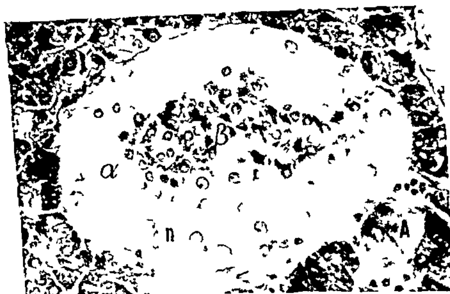


Fig 5

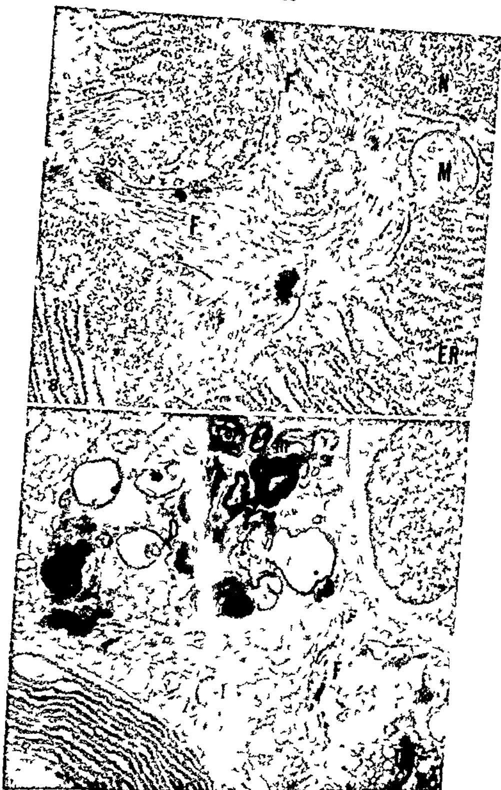
Pancreatic islet from *Chinese hamster treated with* ethionine during 10 days containing central β cells (β) There are large peripheral α -cells (α) exhibiting large nuclei and distinct nucleoli and well demarcated cytoplasm Swollen and necrotic cells (n) probably α -cells also occur In the exocrine portion there is swelling and decreased staining affinity of some acinar cells (A) Aldehyde fuchsin stain × 300

Many cells showed complete loss of basal basophilia and there were cells with eosinophilic bodies in the cytoplasm In the acini there were cells of varying size and staining affinity as well as disintegrated cells In the acinar lumina there were dark masses and eosinophilic bodies At 7 days there were irregular acini with dilated and irregular lumina (Fig 3) that sometimes were filled with dark masses and light amorphous material Atrophy of the acinar parenchyma and replacement by adipose and fibrous tissue occurred mainly at 10 and 14 days In the atrophied parenchyma unaffected acini could be seen Infiltration of inflammatory cells and interstitial oedema were found in areas of atrophy (Fig 4)

In the ducts and ductules there were at all observation times dark masses and occasionally light amorphous material was found in the ductules In areas of atrophy there were numerous ductules and a moderate amount of ducts Some of these appeared to be dilated In the ducts there were sometimes single or multiple goblet cells The ductule cells were unaffected The only alteration observed in the duct cells was the occasional occurrence of crystalline material

The *vessels* in the exocrine pancreas sometimes showed rather thick basement membranes The *nerves* were unaffected

In the *islets* the β cells often showed degranulation At 10 and 14 days there were a few islets with changes of the α_2 cells (Fig 5) Thus


F
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There were numerous cytoplasmic bodies of varying size and appearance In cells with or without other changes there were bodies consisting of fibrillar elements with varying arrangement (Fig 8) These were tentatively called fibrillar bodies Enclosed within them there were sometimes small electron dense rounded or irregular particles The fibrillar bodies occasionally seemed to be connected with mitochondria Membrane-enclosed fibrillar bodies were also encountered

In addition to these cytoplasmic bodies there were more electron dense and irregular structures of varying size Myelin figures and focal accumulations of degenerated cellular components often occurred (Fig 9) There was also necrosis of whole cells

The architecture of the acini was altered There were areas with acinar atrophy and replacement by adipose and fibrous tissue In the acinar lumina there were fibrillar material moderately electron dense amorphous masses and structures suggestive of being degenerative cellular components Thus these structures showed varying electron density and contained particles reminiscent of membranes and granules

Duct system The lumina of the duct system contained moderately electron dense amorphous masses There were apical blebs in the ductule cells Crystalline material was occasionally found in the duct cells (Fig 10) In the ducts there were sometimes goblet cells (Fig 11) In a few of these there was also crystalline material

Pancreatic islets Degranulation of the β cells was often found from the second day At 10 and 14 days there were a few islets with changes of the α cells Thus some of these cells showed prominent nucleoli well developed endoplasmic reticulum and Golgi complex as well as numerous mitochondria and secretory granules Other α cells disclosed swelling and disintegration of the mitochondria and cytoplasmic vacuolization There were also cells with marked degenerative changes with disintegration of the cytoplasm and the cellular organelles (Fig 12) The α_1 cells and the agranular cells were unaffected Sometimes the islet capillaries showed rather thick basement membranes

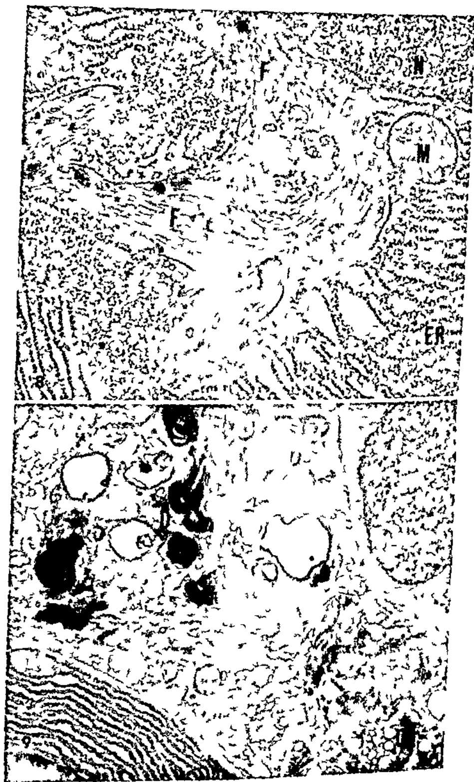
DISCUSSION

Many of the studies with ethionine have utilized animals fed stock or special diets for periods of days to weeks One of the most obvious

Figs 8–9

Fig 8 Portion of acinar cell of hamster after 4 days of ethionine treatment showing fibrillar bodies (F) containing small electron dense particles endoplasmic reticulum (ER) a mitochondrion (M) and nucleus (N) × 34 000

Fig 9 Pancreas from Chinese hamster treated with ethionine during 7 days showing an acinar cell with accumulations of irregular dense vesicular or membraneous structures (D) some of these are myelin like (m) There are also fibrillar bodies (F) × 16 000


F
N
M
E
ER
8
9

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DISCUSSION

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Fig 8 [illegible] fibrillar [illegible] days of ethionine treatment showing [illegible] reticulum [illegible] fine particles endoplasmic [illegible] (N) × 31 000

Fig 9 Pancreas from [illegible] an acinar cell [illegible] ethionine during 7 days showing [illegible] structures [illegible] dense vesicular or mem[illegible] fibrillar bodies [illegible] are myelin like (m) There are als[illegible]

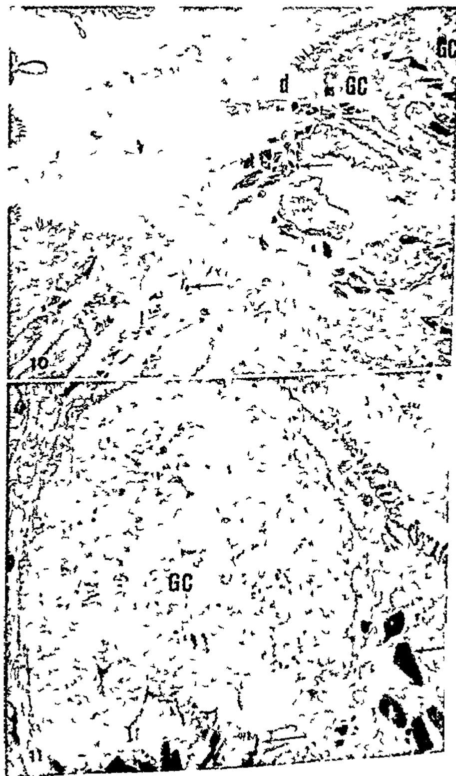

GC
d
GC
10
GC
11



Fig 12

Peripheral portion of pancreatic islet fr m Chine e hamster treated with ethionine during 10 days showing cells with degenerative changes (n) In some of these cells there are secretory granules of α_2 type (α) There is one cell with sparse granulation probably a degranulated β cells (β) in which there are no obvious degenerative changes A capillary (C) with thick basement membrane (BM) is also seen $\times$ 3 000

uncertainties in many such studies is the large and variable decrease in food intake of animals given ethionine as compared to control animals (cf *Farber* 1967) If ethionine is given parenterally it seems to be more easy to get proper dosage than if it is given in the diet In rats no clear cut difference is found whether or not ethionine is administered

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Figs 10–11

Fig 10 Pancreatic duct of Chinese hamster treated with ethionine during 7 days demonstrating duct cells which contain electron dense particles apparently of crystalline nature (arrows) In the duct there are also goblet cells (GC) In one of the e the contents are discharged (d) into the lumen through the ruptured cell membrane $\times$ 3000

Fig 11 Portion of pancreatic duct of hamster after 7 days ethionine exposure showing a goblet cell (GC) In this cell as well a in the neighbouring duct cells there is electron dense apparently crystalline ... $\times$ 17 000


GC
d
GC
10
GC
11

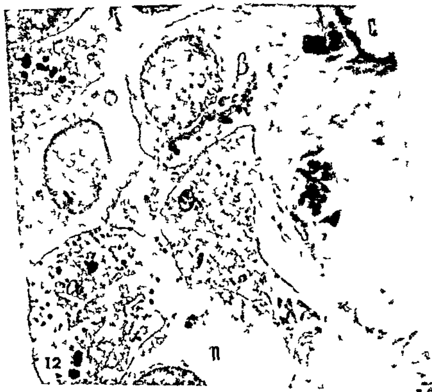


Fig 12

Peripheral portion of pancreatic islet from Chinese hamster treated with [illegible] during 10 days showing cells with degenerative changes (n). In some of these [illegible] there are secretory granules of α type (α). There is one cell with sparse [illegible] probably a degranulated β cells (β) in which there are no obvious degen[illegible] changes. A capillary (C) with thick basement membrane (PM) is also seen. × 3[illegible]

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Fig 11 Portion of pancreatic duct of hamster after 7 days ethionine exposure showing a goblet cell (CC). In this cell as well as in the neighbouring duct cells there is electron dense apparently crystalline material (arrows) × 17 000

directly into the blood stream (*Loring & Hartley* 1955) Intraperitoneal injections have been used in the rat for investigations of the effects on the pancreas by ethionine (*Loring & Hartley* 1955 *Herman & Fitzgerald* 1962) If animals receive ethionine during several days or longer the results are difficult to interpret because of the ability of the organism to adapt readily to this compound within a period of 2 or 3 weeks (cf *Farber* 1967) On the other hand *House et al* (1963) state that one to three weeks are required for complete action of ethionine in the golden hamster The time factor is important in the interpretation of the significance of experimental observations with ethionine (*Farber* 1967) In studies of the effect of ethionine on ribonucleic acid synthesis in the liver it has been found that there is a fall in the incorporation of labelled precursors into RNA in acute experiments (*Farber et al* 1964) but a stimulation of RNA synthesis in long time experiments (*Turner & Reid* 1964) There are differences in the effects if ethionine is administered prolonged or in a large single dose (*cf Meldolesi* 1967) In the present work it was thought most appropriate to give the ethionine as daily intraperitoneal injections By the use of various observation times it was hoped that difficulties in the interpretation because of the time factor would be diminished

The experiments show that the administration of ethionine to the Chinese hamster evokes marked changes of the exocrine pancreas Among these changes those exhibited by the endoplasmic reticulum were conspicuous and early occurring It has been postulated that alterations of the endoplasmic reticulum belong to the earliest and most characteristic in ethionine treated animals (*Ekholm et al* 1962 *Herman & Fitzgerald* 1962) Reduction of the number of ribosomes and altered organization of endoplasmic reticulum have been thought to be consistent with an involvement with RNA and protein metabolism (*Herman & Fitzgerald* 1962) and to be equivalent to the diminution of basophilic substance observed in the light microscope (*Ekholm et al* 1962)

There are different opinions as to the effect of ethionine on the zymogen granules Thus there are descriptions of increased (*Farber & Popper* 1950 *Edlund* 1962) or decreased (*Goldberg & Chaikoff* 1951) number of these organelles The results of the present study seem to conform to those obtained by the latter authors The structures interpreted as prozymogen granules showed membrane discontinuities conforming to those seen in pancreatic acinar cells of rabbits (*Shapiro & Lazarus* 1967)

Fibrillar bodies were often encountered The nature of these is not known but it seems probable that they may be of degenerative nature and possibly originate from endoplasmic reticulum or mitochondria In acinar cells of chicken cytoplasmic inclusions consisting of parallel tubular elements have been found (*di Stefano* 1967) Though these inclusions apparently not exactly conform to the fibrillar bodies

it is known that these cytoplasmic inclusions may occur in association with mitochondria and endoplasmic reticulum

Necrosis of whole acinar cells occurred in the ethionine treated hamsters There were also focal accumulations of degenerated cellular material including myelin structures This may denote degradation of part of a cell with possibilities for resorption or extrusion of the degenerative material (cf *Swift & Hruban* 1964) If so it seems that acinar cells with limited lesions may survive the effects of ethionine and possibly regenerate

The nature of the crystalline material sometimes seen in the duct cells is unknown and remains to be clarified The significance of the goblet cells in the ducts is not clear Though they have not been observed by us in the ducts of normal Chinese hamsters it cannot be excluded that they sometimes may occur normally in this species *Lev & Spicer* (1965) state that goblet cells in man only appear in the pancreatic ducts as they approach the duodenum As goblet cell metaplasia is found in damaged pancreas mainly in areas of scarring (*Walters* 1965) it seems however that the occurrence of goblet cells in the present study may be regarded as a secondary phenomenon to the ethionine induced lesions

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The significance of the seemingly haphazard distribution of the ethionine induced lesions with unaffected and atrophic acini close to each other is not known Such variations have been found also in ethionine treated rats (*Herman & Fitzgerald* 1962) It has been pointed out by *Fitzgerald* (1960) that it is very difficult if at all possible to destroy all acinar cells by ethionine and that these cells may survive even high doses of this compound

In the rat liver there is a sex difference in the response to ethionine and there is considerable evidence that the male rat metabolizes methionine and ethionine somewhat more slowly than does the female (cf *Farber* 1967) Sex differences as to the effect of ethionine on the pancreas in the golden hamster have been recorded (*House et al* 1963) In the present study sex differences were not found

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SUMMARY

DL-ethionine was administered intraperitoneally at a dose level of 0.5 g/kg body weight to 30 Chinese hamsters. Twelve animals injected with saline were used as controls. At pre-determined intervals the animals were sacrificed and specimens were taken from the pancreas for light and electron microscopic examination of degenerative changes.

In the light microscope the acinar cells showed decrease of basal basophilia, cytoplasmic vacuoles and eosinophilic bodies. The acinar lumina were dilated and irregular and contained light or dark masses and eosinophilic bodies. There was atrophy of the acinar parenchyma with replacement by adipose and fibrous tissue. Ultrastructurally the acinar cells demonstrated dilatation of the endoplasmic reticulum, decreased number of ribosomes and zymogen granules, disintegration of the mitochondria, as well as the occurrence of cytoplasmic fibrillar bodies. Focal accumulations of degenerated cellular components and necrosis of whole acinar cells were found. In the ducts there were goblet cells and occasionally crystalline material occurred in the duct cells. Degranulation of the β cells was found in the pancreatic islets. In a few islets some α cells showed signs of increased activity whereas others disclosed marked degenerative changes. The α_1 cells and the agranular cells were unaffected. Thick basement membranes were sometimes seen in the vessels of the exocrine and endocrine pancreatic parenchyma.

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TABLE 1
Vaccination History of 324 Pre School Children

Children born in	Year of primary immunization against poliomyelitis																		Vaccination		Total
	1961			1962			1963			1964			1965			1966			not performed	uncertain	
		3	4	2	3	4	2	3	4	2	3	4	2	3	4	2	3	4			
1960	1	13	23	2	22	3		(			2			2		1				3	77
1961		21			25	4		28	2		6					3			1	5	4(
1962					3		3	23		1	25			4					1	1	62
1963											2/		4	18		1			1	5	56?
1964										1	2		)	34		8			1	1	53
Number of Injections																					324

with imported inactivated vaccine. During the following 8 year period the vaccinations were extended to all individuals. Since 1958 solely inactivated vaccine manufactured in Sweden has been employed.

The vaccination course recommended runs as follows. Two injections of 1 ml of inactivated vaccine are given 1 to 2 months apart. A third injection is administered 1 year after and a fourth 5 years after the first two doses.

Plan of the Study

Altogether 27 day nurseries, play schools and first grade school classes in Stockholm city were enroled. These comprised close to 1100 children aged 2 to 7 years.

Questionnaires were distributed to the parents of the children. Distribution and collection was carried out by aid of the personnel at the different units.

The questions concerned the following: How many poliovirus vaccine injections has the child received? In which years were they performed? Do you consider your data correct, probably correct or uncertain? Do you consent to finger puncture of your child for blood sampling?

Random blood samplings were made from 324 pre-school children (2 to 6 years old) whose parents had consented to it. From the 7 years old school children the first 100 samples were randomly collected; later an additional number (76) were taken from children who had received 3 doses of vaccine only.

Altogether 500 samples were investigated for antibody content. All were tested for type 1 and type 3 poliovirus antibodies. Only 104 samples were tested for type 2 antibodies.

RESULTS

Responses to Questionnaires

Almost 100 per cent of the questionnaires were answered and returned. Ninety seven per cent claimed that their child had been immunized to poliomyelitis, 2 per cent were reported as unvaccinated and 1 per cent did not remember whether or not the child was vaccinated. Eighty nine per cent claimed that their answers were correct (or probably correct) while the rest, i.e. 11 per cent were uncertain. Ninety eight per cent consented to blood sampling.

Results of Antibody Determinations. Antibody Levels in Pre-School Children in Relation to Their Year of Birth

The serologically tested pre-school children were arranged according to year of birth and vaccination history (Table I). The data from the children whose vaccination data had been claimed to be correct or probably correct are described in detail. Table 1 shows that in general 50 per cent of the children had received the first 2 doses of vaccine in the calendar year following that of birth while the majority of the others received them one year later.

The distribution of antibody levels in the age groups 2 to 6 years are illustrated by immunologic profiles in Fig. 1. Only antibody levels against poliovirus type 1 and 3 are shown. The geometric mean titres in the different age groups are shown in Fig. 2. The type 2 antibody levels are here also included. It can be seen that the antibody distribution appears to be fairly similar in the groups tested; also the mean levels do not diverge to any noticeable extent. Generally slightly

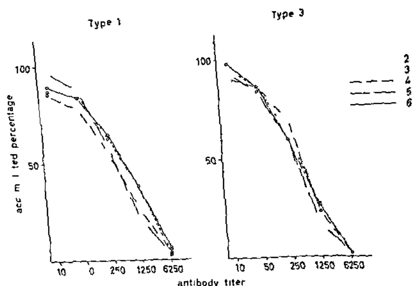


Fig 1

Immunologic profiles i e accumulated titre distribution of different age groups of pre s hool children The vertical axis gives the accumulated percentage i e the p rcentage of children attaining or surpassing indicate l titre The horizontal axis gives the antib dy titre expressed as the recipro al of the last neutralizing serum dilution The different age groups are indicated in the figure

lower mean titres in the 4 to 5 years old children can be noticed however

Sero-Negative Samples

Among the pre school children studied 9 per cent were found to be sero negative to type 1 and 6 per cent to type 3 in the lowest dilution tested i e a dilution of 1 10 The results are shown at the bottom of Fig 2

Antibody Levels in Relation to Information about Vaccination History

The antibody data were further analysed according to the year of the first injection and the number of injections received The results are illustrated by immunologic profiles in Figs 3 and 4 and by mean titres in Fig 5 Fig 3 shows the antibody distribution after 3 injections The left part of the figure represents type 1 antibody levels and the right part the type 3 levels

It can be noticed that the type 1 titres in the children vaccinated for the first time in the years 1960 and 1961 generally are lower than titres in those vaccinated later The type 1 titres of children vaccinated between 1962 to 1964 show similar distributions however

The type 3 antibody profiles do not display any noticeable difference between the children vaccinated during the years 1960 to 1964

with imported inactivated vaccine During the followi... tions were extended to all individuals Since 1959 ... factured in Sweden has been employed

The vaccination course recommended runs as foll... inactivated vaccine are given 1 to 2 months apart A t... 1 year after and a fourth 5 years after the first two ...

Plan of the Study

Altogether 27 day nurseries play schools and first grad... holm city were enroled These comprised close to 1100 chi...

Questionnaires were distributed to the parents of the ch... collection was carried out by aid of the personnel at the diff...

The questions concerned the following How many poliom... has the child received? In which years were they performed? I... data correct probably correct or uncertain? Do you consent t... your child for blood sampling?

Random blood samplings were made from 324 pre school chil... old) whose parents had consented to it From the 7 years old s... first 100 samples were randomly collected later an additional n... taken from children who had received 3 doses of vaccine only

Altogether 500 samples were investigated for antibody content A... for type 1 and type 3 poliovirus antibodies Only 104 samples were te... antibodies

RESULTS

Responses to Questionnaires

Almost 100 per cent of the questionnaires were answered ... turned Ninety seven per cent claimed that their child had bee... munized to poliomyelitis 2 per cent were reported as unvaccin... and 1 per cent did not remember whether or not the child was va... nated Eighty nine per cent claimed that their answers were corre... (or probably correct) while the rest i e 11 per cent were uncertain Ninety eight per cent consented to blood sampling

Results of Antibody Determinations Antibody Levels in Pre School Children in Relation to Their Year of Birth

The serologically tested pre school children were arranged according to year of birth and vaccination history (Table 1) The data from the children whose vaccination data had been claimed to be correct or probably correct are described in detail Table 1 shows that in general 50 per cent of the children had received the first 2 doses of vaccine in the calendar year following that of birth while the majority of the others received them one year later

The distribution of antibody levels in the age groups 2 to 6 years are illustrated by immunologic profiles in Fig 1 Only antibody levels against poliovirus type 1 and 3 are shown The geometric mean titres in the different age groups are shown in Fig 2 The type 2 antibody levels are here also included It can be seen that the antibody distribution appears to be fairly similar in the groups tested also the mean levels do not diverge to any noticeable extent Generally slightly

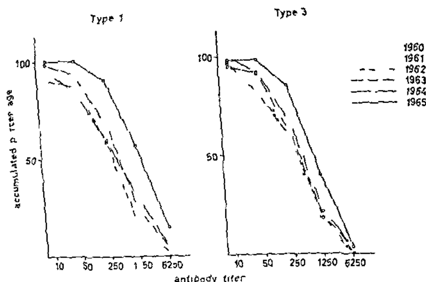


Fig 3

Immunologic profile of children having received 3 *injections of poliovirus* vaccine The year when the two first *injections were administered is indicated in* the figure Other symbols as in Fig 1

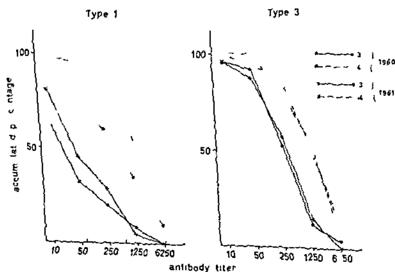


Fig 4

Immunologic profiles" of children having received 3 and 4 doses of vaccine beginning in 1960 and 1961 Symbols as in Fig 1

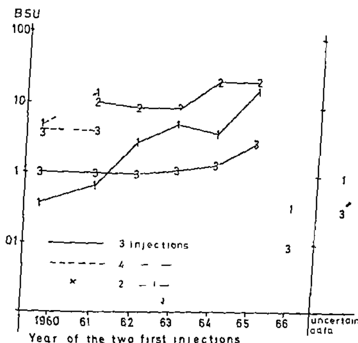


Fig 5

Geometric mean titres (B S U) of serum samples in relation to number of injections and time of the first two injections Symbols as in Fig ?

children whose data were recorded as uncertain or whose vaccinations were incomplete (*i e* less than 3 injections in 1965 or earlier)

Generally high type 2 mean titres can be observed In the children inoculated twice within the last year low titre levels against all three types were generally found The group in which records were uncertain also displayed means below the general levels

Vaccination Record of Sero Negative Children

Fourteen children out of the 500 tested were found to be sero negative to both types of virus investigated With one exception they were reported as unvaccinated (7) not fully vaccinated (3) or the parents were uncertain of the number of injections given (3)

DISCUSSION

Answers to Questionnaires

Ninety seven per cent of the children questioned were reported to be vaccinated against poliomyelitis Since 1960 all children have been offered vaccination at the Well baby clinics Over ninety per cent of Swedish children are regularly brought to these clinics during their first years of life The high frequency of vaccinated children thus

affirms that the system of enrolment for poliovirus vaccination is satisfactory

Eighty nine per cent of the parents claimed that the information given was correct This was a surprisingly high figure No official registration of the names of the individual vaccinees is undertaken However every mother is presented with a health card for her baby at the maternity hospital and she is supposed to register all vaccinations on this card She may also obtain some information from the Well baby clinic Still the data may not be as correct as it would be under controlled experimental conditions The vaccination data (Table 1) of the various age groups follow a very similar pattern which favours the assumption that the reported data in general may be correct

Antibody Patterns in Relation to Age

The antibody titre distribution in the age groups 2 to 6 years were found to be almost congruent This similarity in itself must be regarded as satisfactory Thus the vaccination programme followed in Sweden apparently has succeeded in inducing an even level of protection in the investigated age groups at the time of this sampling

It must be stressed that no cases of poliomyelitis have been reported from the area of investigation since 1960 nor has any poliovirus been isolated (3 4) In the country as a whole an epidemic with 50 paralytic cases occurred in Gothenburg in 1961 Since that time none or 1–2 cases (imported ones) per year have been registered Thus the influence of natural infection on any of the results presented here can be disregarded

Antibody Patterns in Relation to Year of Primary Vaccination

The discrepancy between type 1 and type 3 antibody levels in relation to the date of vaccination is noteworthy The dissimilarity concerns only children receiving their primary vaccination in 1960 and 1961 however Thereafter the type 1 and type 3 antibody titres follow a very similar pattern The immunogenic potency values of the Swedish inactivated vaccines had been tested yearly in triple negative children (2 7) Fig 6 shows the yearly means of antibody levels in triple negative children tested 2 weeks after 2 injections of inactivated vaccine According to these tests titre levels to type 1 were generally found to be lower in the years 1957 to 1961 than in the following years This change of immunogenicity has been analysed in a previous paper (2) and was found to be coincidental with a change of the seed strain used for vaccine production from the type 1 Stockholm 53 strain to the Brunenders strain It can also be noticed in the figure that since 1962 the type 1 levels have been similar to the high type 2 and type 3 post vaccination antibody levels which had been reached since 1958 and

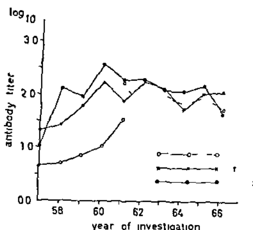


Fig 6

Results of potency tests of inactivated vaccine performed in triple negative children The vertical axis gives the mean titres of the children tested the horizontal axis the year of investigation The type 1 mean titres are illustrated by a whole drawn line when the Stockholm type 3 strain was used as the seed strain for the vaccine The dashed line represent the titres to appear when the Brunenders strain was used

1959 respectively Thus the results of the present investigation are in full agreement with the outcome of the vaccine tests This fact also favours a high validity of the vaccination histories reported

Remarks on Duration of Immunity

Of further interest in Fig 3 and Fig 5 are the remarkably similar antibody levels to type 2 and 3 in children vaccinated from 1960 to 1964 and to type 1 from the years 1962 to 1964 Only the vaccinees who had completed their vaccination with a third dose within the last year of this investigation showed higher levels to all types Since the vaccine potency had not varied considerably in the years mentioned above the results of this investigation suggest that the titres remain fairly stable after the first post vaccination fall of titre has occurred during the year following the vaccination Although this phenomenon was observed by Salk (6) in a few cases continuously investigated over extended periods of time it has not been supported before by large scale investigation On the contrary the supposition that antibodies induced by immunization with inactivated vaccine should be less persistent than those raised by vaccination with live attenuated virus has been almost generally accepted

The type 2 titres are generally high which is in accordance with all experience in this field Previous studies by the author have indicated that the kinetics of the antibody response to type 2 antigen differs from that of type 1 and 3 (2) The type 2 antibodies appeared to increase in titre for a longer period of time Thus the levels were generally higher in samples collected 7 months after the 2 primary

injections than after 2 weeks a phenomenon never observed in the case of the other two virus types

The results illustrated in Fig 5 do not contradict this observation The general postvaccination titre levels of type 2 antibodies declined later than those of type 1 and 3

The Effect of a Second Booster (the Fourth Dose)

An evaluation of the titre levels in children having received 3 or 4 doses (the latter having received a second booster) suggests (Fig 5) that the effect of the late booster is correlated to the vaccinees immunity status prior to the booster

SUMMARY

The poliovirus vaccination history of close to 1100 children mainly pre school children was collected in Stockholm in the autumn of 1966 According to the information given by the parents 97 per cent had been vaccinated Incomplete vaccinations i e less than 3 injections administered in 1965 or earlier were reported from 3 per cent of these Eighty nine per cent claimed that the vaccination history given was correct

In 324 randomly selected pre school children antibody titrations showed a similar titre distribution in all pre school age groups A total of 9 per cent was sero negative to poliovirus type 1 in the lowest serum dilution tested i e 1 10 The corresponding figure for type 2 and type 3 was 1 and 6 per cent respectively

Analysis of titres in relation to the date of vaccination suggested a good correlation between previously routine vaccine potency tests performed in children and levels observed in the presently investigated children The children reported to be primarily vaccinated after 1961 showed demonstrable antibodies of the same magnitude to all three types of poliovirus over 95 per cent had demonstrable antibodies in the dilution 1 10

The results also support the assumption that the antibody levels remain fairly stable after the main post vaccination fall has occurred in the year following the immunization

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EXPERIMENTAL STAPHYLOCOCCAL ARTHRITIS IN GOLDEN HAMSTERS (MESOCRICETUS AURATUS)

By

LARS LINDBERG

Received 17.x.68

Investigation of the course of the tissue reaction and other features of an infection often requires model experiments in laboratory animals. Search of the literature for experimental staphylococcal arthritis in small laboratory animals, however, fails to reveal more than a few investigations in which such infections have been induced. Several relevant papers on staphylococcal arthritis in the rabbit are available (*Rigdon* 1942, *Frankel et al.* 1943, 1944 and 1958, *Davis & Ring* 1960, *Bardenheier et al.* 1966) but reports concerning such lesion in the *guinea pig*, albino rat and mouse are not available, possibly because of the difficulty in inducing staphylococcal arthritis in these animals. The *golden hamster* seems not to have been tried in this respect. In certain experiments it is very difficult, sometimes even impossible, to use an animal as large as the rabbit in investigations requiring very large series of animals or in pharmacokinetical studies using expensive substances such as radioactively labelled antibiotics (*Lindberg & Lundberg* 1969). The purpose of this investigation therefore was to devise a method for producing a model infection with *Staphylococcus aureus* in a joint of a laboratory animal smaller than the rabbit.

MATERIAL AND METHODS

Choice of Bacterial Strain

Staphylococcus aureus was used. As the pathogenicity can be expected to vary from strain to strain, eight strains with positive coagulase reaction against guinea pig plasma at 37° C were selected for examination of their pathogenicity for the guinea pig and the golden hamster. The phage types are given in Table 1. Suspensions of each strain were prepared in the following way.

From the blood agar dish where the strain had been grown, one platinum loopful of the culture was transferred to a test tube containing 4.5 cc of broth and thoroughly homogenized. The broth consisted of: Bacto beef extract 0.5 per cent, Bacto peptone 1 per cent, NaCl 0.3 per cent, $Na_2HPO_4 \cdot 2(H_2O)$ 0.2 per cent, distilled water 98 per cent. The pH of the medium was 7.6–7.8. The test tube was incubated for eighteen hours at 37° C. The amount of the bacteria in the broth was there-

This investigation was made possible by a grant from Alfred Österlunds Stiftelse

after standardized photometrically by dilution with physiological saline The number of bacteria was 10^9 per cc as judged by dilution series culture and colony counts

Each bacterial strain was tested by intra articular injection of 0 1 cc of suspension into the left knee of two guinea pigs and by injection of 0 05 cc into the left knee of two golden hamsters The animals were observed daily for four weeks for any general or local reaction A slight swelling of the knee joint during the first 1–5 days was the only reaction found in the guinea pigs and a clear swelling lasting throughout the four weeks indicating active infection was observed in all golden hamsters

TABLE 1

Strain	Phage type	Group
1518	29+	I
1596	non specific 52/52A/80	I
P 3844	29+ (79/6557)	I
4432	3 A	II
5843	7/47/54/75/83A/3945/6557	III
5885	3 A	II
5924	"	III
Wood 46	NT	

As the strains did not differ from one another in pathogenicity the well known strain Wood 46 was chosen for the continued investigation The strain had been kept on blood agar dishe at + 4 C and had been subcultured once a month when also the phage type and antibiogram had ben checked

Animal Experiment

For this investigation seventeen golden hamsters weighing about 100 grams were used 0 05 cc of the bacterial suspension was injected into the left knee of each golden hamster The animals were sacrified at predetermined intervals (Table 2) The infected knee joint was removed fixed in 10 per cent formalin decalcified in formic acid embedded in paraffin Histological sections 7 microns thick were cut and stained with haematoxylin eosin or according to van Gieson

TABLE 2

Number of animals	Killed after
3 + 1S	3 days
4	1 week
3	2 weeks
2	4 weeks
2	6 weeks
2	8 weeks

S = succumbed during experiment

RESULTS

Three days Owing to postmortem autolysis the animal that died spontaneously was excluded from the analysis In two of the other animals the joints showed moderately advanced arthritis (Fig 1) The joint cavity contained inflammatory cells The synovial membrane was masked by inflammatory changes The joint capsule and the extra articular tissue contained inflammatory exudate with numerous polynucleated cells



Fig 1
Hamster knee three days after injection of *Staphylococcus aureus* Incipient abscess formation dorsally in the knee fold and in the suprapatellar recess Dorsally the muscles show infiltration of inflammatory cells
van Gieson 115 ×



Fig 2
Hamster knee three days after injection of *Staphylococcus aureus* Infection has progressed more rapidly than in the joint in Fig 1 Joint cartilage and bone trabeculae in both epiphyses are partly destroyed van Gieson 115 ×

This exudate extended down along the muscles of the lower leg The joint cartilage the epiphyseal lines and the bone marrow of both the tibia and the femur were of normal appearance The nuclei in some of the osteocytes in the bone near the joint were pycnotic

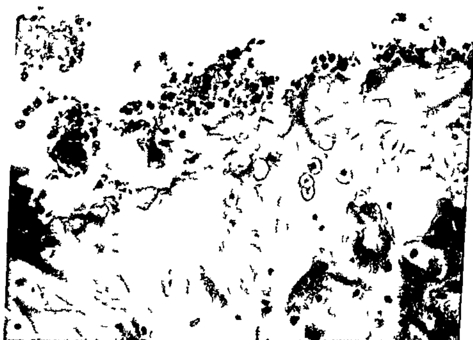


Fig 3
Destruction of joint cartilage three days after injection of *Staphylo ccus aureus* van Gie on 400 ×



F j 5
Ham ter knee one week after injecti n f *Staphyl c us aureus* l rge al c c in ventral and d rsal part of joint. Fem ral epiphysis partly destr ye l c ntrally van Gie n 11 5 ×



Fig 5
Hamster knee two weeks after injection of *Staphylococcus aureus* van Gieson 11 5 ×

In the joint of the remaining animal the infection was more advanced (Fig 2) The joint cavity and capsule as well as the periarticular tissue were penetrated by pus The muscular interspaces in the thigh contained inflammatory cells The joint cartilages were partly eroded (Fig 3) Necrotic cells were seen in the epiphyseal lines and in several of the bone trabeculae of both epiphyses The entire marrow of the femoral epiphysis was necrotic

One week In three of the animals the infection was more advanced than after three days and the joint cavity was distended by a large amount of pus (Fig 4) All soft tissues in and around the joint contained numerous polynucleated leucocytes The articular cartilages were partly destroyed especially on the ventral aspect of the femoral epiphysis Smaller parts of the epiphyseal lines were necrotic Abscesses were seen in two of the tibial epiphyses In several osteocytes in trabeculae near the joints the nuclei were pycnotic

In the remaining animal the gross anatomical topography was un



Fig 6
Complete destruction of hamster knee two weeks after injection of *Staphylococcus aureus* van Gieson 11 5 ×

changed but the whole joint including the cartilage bone marrow of both the femoral and the tibial epiphyses and metaphyses were totally necrotic Small scattered groups of polynucleated leucocytes were seen

Two weeks In one animal the picture resembled that seen after one week but the amount of pus in the joint cavity had decreased as had the infiltration of inflammatory cells in the periarticular tissues The ventral part of the femoral epiphysis was destroyed (Fig 5)

In the remaining two animals the joints were totally destroyed Only remnants of the epiphysis and the epiphyseal lines could be recognized (Fig 6) The region between the bone ends was occupied by a granulation tissue rich in cells with scattered polynucleated leucocytes partly aggregated to small abscesses

Four weeks In one animal the infection was regressing in that only some parts of the joint capsule and periarticular tissue contained inflammatory cells The joint cartilage ventrally on the femoral epiphysis was eroded and in other areas it was necrotic The epiphyseal line the bone marrow and the bone tissue were of normal appearance



Fig 7

Hamster knee six weeks after injection of *Staphylococcus aureus* Infection healed after partial destruction of the femoral epiphysis van Gie on 11 5 ×

In the remaining animal the picture was the same as in the animal illustrated in Fig 5

Six weeks In both animals the infection appeared to have healed and no pus or inflammatory cells was seen

In one of the animals the infection had healed without demonstrable sequelae In the other the joint cartilage of the femur was partially eroded and there the joint capsule had become firmly attached to the bone (Fig 7) In other areas the joint cartilage was necrotic and contained no stainable cells but the morphology of the joint was otherwise preserved

Eight weeks In both of the animals the infection had healed with complete destruction of the joint

In one animal the joint was destroyed and the area between the bone ends was full of a thick fibrous tissue (Fig 8) In the other animal the subsynovial connective tissue was thickened and the cartilage was partly necrotic and partly eroded with the result that the sub



Fig 6
Complete destruction of hamster knee two weeks after injection of *Staphylococcus aureus* van Gie on 11 5 ×

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sumption also strengthened by personal unpublished and unsuccessful experiments

In a search for another laboratory animal suitable for this kind of model infection the mouse proved too small to allow satisfactory intra articular infection and it therefore seemed reasonable to try the golden hamster which has apparently not been used before for this purpose

Results

Purulent arthritis developed in most of the animals (in 14 or 15 of 17) It reached a maximum after one week when large amounts of pus were seen in and around the joint Signs of incipient regression appeared after about four weeks and after 6–8 weeks no certain signs of infection were any longer demonstrable

The final picture varied with the severity of the arthritis from complete or partial recovery (Fig 7) to complete (Fig 8) destruction of the joint

Only one of the seventeen animals succumbed during the experiment In view of the high frequency of deaths otherwise claimed when the rabbit has been used for infections of bone and joints (*Lindberg* 1967) this low loss in the present investigation underlines the usefulness of the golden hamster

Judging from the observations set forth above the golden hamster is a suitable animal for infections of the joints with *Staphylococcus aureus*

SUMMARY

A method is described by which it is possible to induce infectious arthritis in golden hamsters by intra articular injection of *Staphylococcus aureus*

All animals displayed purulent arthritis Signs of healing were seen after 4 weeks After 6–8 weeks the infection was healed with extensive destruction of the joints as end result

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THE DISTRIBUTION OF TRITIUM LABELLED DIHYDROSTREPTOMYCIN AND TETRACYCLINE IN STAPHYLOCOCCAL ARTHRITIS

An Autoradiographic Study of Golden Hamsters and Rabbits

By

LARS LINDBERG and BO LUNDBERG

Received 28 x 68

Antibiotics are the most important therapeutics used in the treatment of bacterial arthritis Such therapy however often fails to ward off partial or complete destruction of affected joints It appears that the substance given does not reach or does not affect the bacteria in certain parts of the focus

The purpose of the present investigation was to assess the distribution of dihydrostreptomycin and tetracycline in an infected joint to find out whether any parts of the infected joint are not reached by these antibiotics

In a contemplated series of studies other antibiotics will also be tried The distribution of antibiotics in various body tissues is conventionally assessed by bacteriological determination of the amount of antibiotic in biopsy specimens This method however is relatively crude and does not take into account the fact that different tissues in the biopsy specimen are intermingled and that the pieces of tissue removed often are contaminated with blood and tissue fluid containing antibiotics in high concentration In this investigation a method is used by which it is possible to avoid the above mentioned sources of error of the antibiotic in the various tissues of the infected joint The investigation was carried out on animals with experimental arthritis using an autoradiographic technique described by *Ullberg* (1954) and *André* (1956) which allows location of water soluble substances Since the route of administration of these substances is of interest also clinically the distribution of the antibiotics in the joint was studied both after parenteral and intra articular administration

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This investigation was made possible by a grant from *Alfred Österlunds Stiftelse*

MATERIAL AND METHODS

Use was made of tritium labelled dihydrostreptomycin sulphate with a specific radioactivity of 2 96 mCi/mg and a radiochemical purity of more than 90 per cent and tritium labelled tetracycline with a specific radioactivity of 41 8 mCi/mg and a radiochemical purity of more than 97 per cent[1]

For the sake of economy the distribution of the antibiotics after parenteral injection was studied in golden hamsters while for technical reasons the distribution after intra articular injection of the substances was studied in rabbits

In the study of the distribution after *parenteral injection* staphylococcal arthritis was produced in golden hamsters by a method described by *Lindberg* (1969) Forty animals weighing about 100 g received an injection of 0 05 ml of an 18 hour broth culture of *Staphylococcus aureus* strain Wood 46 containing about 10^9 colony forming units per ml Two groups of animals with 20 animals in each were used One group received dihydrostreptomycin the other tetracycline Among the animals in the dihydrostreptomycin group 10 were given an injection of the drug 2 days after infection with the bacteria and 10 animals received it 2 weeks after infection with the bacteria The dose consisted of 0 25 ml of dihydrostreptomycin sulphate containing 0 78 mg of dihydrostreptomycin sulphate corresponding to [illegible]3 mCi/animal In the tetracycline group the animals received an injection of 0 25 ml of tetracycline solution containing 0 33 mg of tetracycline corresponding to 13 8 mCi/animal 2 days after the infection with the bacteria and the remaining 10 animals a corresponding injection 2 weeks after the bacterial injection All antibiotics were injected intramuscularly under the left scapula Two animals in each of the four subgroups were killed 15 minutes 30 minutes 1 hour 3 hours and 6 hours respectively after the injection of the antibiotic

In the study of the distribution of antibiotics *injected intra articularly* arthritis was produced by injection of 0 2 ml of the same bacterial suspension (see above) into the shoulder joints of 20 rabbits weighing about 2 kg Dihydrostreptomycin sulphate was used in one series tetracycline in the other either drug being injected intra articularly into 5 animals from each group 2 days after the injection of the bacteria (acute arthritis) the remaining 5 animals in each group received it 3 weeks after (destructive arthritis) the injection of bacteria Each joint received an injection of 0 2 ml containing 0 1 mg of dihydrostreptomycin sulphate corresponding to 300 μCi or 0 007 mg of tetracycline corresponding to 300 μCi The animals were killed at the same intervals as those used for the experiment with golden hamsters with the exception that none was killed at the 30 minutes interval

As soon as the animals had been sacrificed the infected joints were removed and frozen in a mixture of hexane and carbon dioxide snow The knee joints were freeze dried for a week embedded in paraffin and tape sectioned (*André* 1956) The shoulder joints were tape sectioned in —15 C according to *Ullberg* (1954) The tape mounted sections were fastened to Ilford G 5 autoradiographic plates After exposure the strip with the adherent sections and the autoradiographic film were separated the autoradiogram developed and fixed finally the sections were stained with haematoxylin eosin and mounted The autoradiogram and the histological section were afterwards compared by placing the section over the autoradiogram

RESULTS

Parenteral Administration

Neither in the group that received dihydrostreptomycin nor in the one that received tetracycline 2 days after injection of the bacterial suspension did the distribution pattern of the antibiotics differ with certainty from that found when the antibiotic was not given until 2 weeks after the bacterial injection

[1] The tritium labelled dihydrostreptomycin and tetracyclin were obtained from Avdelningen för Isotoptjänst AB Atomenergi Studsvik Sweden
Purified chlortetracyclin and streptomycin sulphate for preparation of the radioactive substances was courteously supplied by AB Astra Sodertalje Sweden

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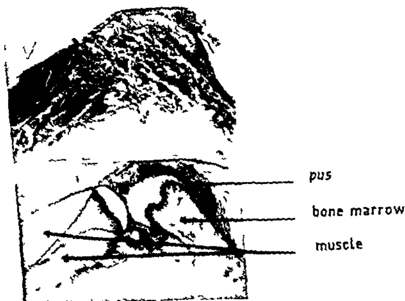


Fig 5

Histological section and corresponding autoradiogram of knee infected two days previously The animal was killed 1 hour after the injection of tritium labelled dihydrostreptomycin Dihydrostreptomycin is still concentrated in the pus filled joint Haematoxylin Magnification × 8

larly injected tetracycline activity was demonstrable only locally in subchondral bone

DISCUSSION

In an planned investigation of a series of antibiotics with the procedure described above *dihydrostreptomycin and tetracycline* were selected first because they are easy to label with tritium and are relatively cheap and second because they are metabolized to only a small extent in the body (*Andre* 1956 *Lindberg* 1967)

Dihydrostreptomycin is no longer used in human medicine because of its occasional side-effects on the auditory nerve It was however used in the present investigation since it apparently has the same pharmacokinetic and antibacterial properties as streptomycin and since it is much easier to label with tritium than streptomycin

Successful autoradiography with the technique used here requires first prevention of redistribution of the water soluble radioactive substance and second histological sections of undecalcified bones With the degrees of microscopical magnification used in the present investigation no redistribution of either antibiotic was observed The possibilities of using higher magnification of the tissue is limited by the fact that the material contains undecalcified bone which is difficult to section

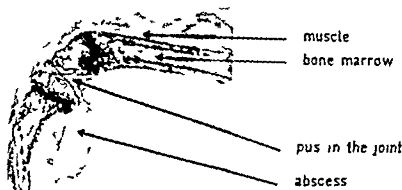


Fig 3

Autoradiogram of knee infected 2 days previously The animal was killed 15 minutes after injection of tritium labelled dihydrostreptomycin Dihydrostreptomycin has not yet entered one abscess in the suprapatellar recess The bad quality of the autoradiogram is due to difficulties in sectioning the non decalcified bone tissue Magnification × 10



Fig 4

Autoradiogram of knee infected 9 days previously The animal was killed 30 minutes after injection of tritium labelled tetracycline Tetracycline has not yet entered an abscess dorsally in knee Magnification × 8

acute arthritis until at least 3 hours after the injection The effect of administration by this route appeared to be confined practically to the joint cavity since no activity of either substance was demonstrable periarticularly (Fig 8) In more severely destroyed joints the distribution both of dihydrostreptomycin and of tetracycline during this period was most complete in abscesses communicating with the site of injection but neither antibiotic had penetrated into abscesses not in open communication with the joint nor had they diffused beyond the borders of the affected joints (Fig 9) At this stage in contrast to findings in acute arthritis the streptomycin persisted 6 hours in infected communicating areas 3 and 6 hours after the injection of the intra articu

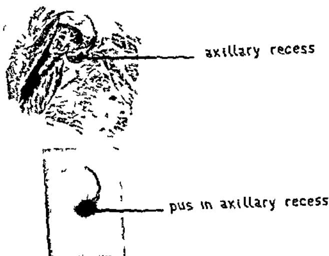


Fig 8

Histol gical section and corresp nding autoradiogram of shoulder joint infected 9 days previously The animal was killed 3 hours after the intra arti ular injection of tritium labelled dihydrostreptomycin The antibiotic is *confined* to the cavity and cartila e of the joint Haematoxylin Magnification × 1

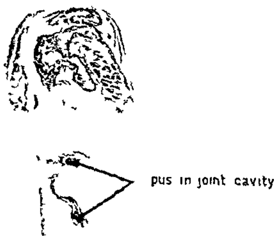


Fig 9

Histological section and corresponding autoradiogram of houlder joint infected 3 weeks previously The animal w s killed 1 hour after the intra articular inje tion of *tritium labelled* dihydrostreptomycin As in acute arthritis the antibiotic is confined to the joint cavity Haematoxylin Magnification × 1

The present investigation would not allow any conclusions about differences in absolute concentration values between *dihydrostreptomycin* and tetracycline because these *two* antibiotics differ considerably in specific radioactivity. Only conclusions about the relative distribution patterns can be drawn.

The difference between the autoradiographic distribution of dihydrostreptomycin and that of tetracycline in healthy joint tissue following *parenteral administration* described by *André* (1956) was seen also in the present study. Dihydrostreptomycin accumulates in mesenchymal *tissues such* as cartilage, periosteum, connective tissue etc. Tetracycline tends to accumulate in bone tissue but is otherwise diffusely distributed. In a few abscesses produced by intramuscular injections of *Corynebacterium pyogenes André* also found the two antibiotics to penetrate the lesions. Here dihydrostreptomycin was even observed in a higher concentration *than that in the surrounding tissue. However* the material was too small to permit any general conclusions. In an investigation with the same technique *Lindberg* (1967) found that the concentration of dihydrostreptomycin was relatively high in tuberculous osteomyelitic abscesses.

Observations made in the present investigation show *that very high* concentrations of dihydrostreptomycin can be achieved in pus in joints *infected with staphylococci*. The accumulation of dihydrostreptomycin in pus occurs rapidly. Already after 15–30 minutes the concentration of dihydrostreptomycin in pus filled joints is much higher than in surrounding soft tissues (*Fig. 1*).

Tetracycline appears to diffuse into the tissues at the same rate as dihydrostreptomycin: after 15–30 minutes tetracycline was found in all tissues except in the central part of one abscess (Fig. 4). However the distribution pattern in the tissues is quite different: the concentration *of tetracycline in the abscesses and in tissue* rich in inflammatory cells was roughly the same as in surrounding tissue, an observation also made by *André* in soft tissue abscesses.

After 3 hours the concentration of both the dihydrostreptomycin and tetracycline in all soft tissues had begun to fall because of elimination *of the substance from the body*, and after 6 hours the autoradiogram showed no signs of antibiotic except in calcified bone where the concentration of tetracycline remained unchanged.

The fact that the concentration of dihydrostreptomycin was *high in* the contents of the abscess while that of tetracycline was not higher than the concentration in surrounding tissue can perhaps be explained on the assumption that large amounts of dihydrostreptomycin but not of tetracycline are reversible bound to certain components of the contents of the abscess (*Lindberg* 1967). The nature of the bond is not clear but it is probably a protein bond. The amount of dihydrostreptomycin bound would than be able to act as a depot from which antibacterially active dihydrostreptomycin is *supplied when the concen*

tration in the serum and surrounding tissues falls owing to excretion of the antibiotic from the body.

Two factors which have been considered in the evaluation of the autoradiographic results of *intra articularly injected antibiotic* are the extent to which it spread in the infected joint and the time the antibiotic persists there after such administration. It is obvious that in acute arthritis the joint cavity is completely filled with antibiotic whether dihydrostreptomycin or tetracycline is used. This distribution can be explained on physical grounds and the antibiotic is evenly distributed in the entire joint cavity. In the animals in which arthritis had persisted for 3 weeks the antibiotics was distributed according to the same mechanism. At this stage of destruction and repair some encapsulated foci however appear inaccessible to intra articularly injected antibiotic. No antibiotic was seen periarticularly. Investigations with the same autoradiographic technique in connection with measurement of the disappearance rate of I^{131} Hippuran and $Na^{22}Cl$ from the shoulder joint of the rabbit showed that already after 15 minutes the same volume corresponding to 10 µCi had spread periarticularly (*Lundberg* 1969). The limited penetrability of the antibiotics studied here is probably due to the largeness of their molecules. The blackened areas over the abscesses even 6 hours after the injection of dihydrostreptomycin indicate the binding of this antibiotic in the abscess. The greatest advantage of administration by the intra articular route is probably that it is possible to achieve a high local concentration of antibiotic. The clinical application of intra articular injection of these antibiotics in arthritis seems however limited especially in the treatment of chronic forms of the disease. Our results suggest that simultaneous parenteral administration is necessary if all affected tissues are to be reached by the antibiotic.

SUMMARY

The distribution of dihydrostreptomycin (for technical reasons used instead of streptomycin) and tetracycline in experimental staphylococcal arthritis has been studied with autoradiographic technique. After intramuscular injection both antibiotics are found to penetrate well into the infected tissues even into abscesses. Dihydrostreptomycin is found to be concentrated in pus compared to normal soft tissues whereas tetracycline is found to be evenly distributed among all soft tissues. The distribution after intra articular injection of the antibiotics is complete but strictly limited to the joint cavity and communicating infectious foci until 3 hours after the injection.

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SUBTYPING OF HUMAN HAPTOGLOBINS WITH PRECIPITATING ANTISERA I

By

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Received 15 x 68

Three common types of human haptoglobin (Hp 1–1 Hp 1–2 and Hp 2 2) can be distinguished on the basis of starch gel electrophoresis patterns (1) *Connell Dixon & Smithies* subjected purified haptoglobin to reductive cleavage and electrophoresis in acid starch gel containing mercapto ethanol and 8 M urea (2) Using this method they found two kinds of polypeptide chain α and β Three types of α chain were distinguishable Hp 2α Hp 1Fα and 1Sα Family studies (3) indicated that these are the expressions of three alleles Hp¹ Hp² and Hp at the Hpα locus The β chain is the same in all the common phenotypes (4) Thus it is possible to divide the Hp 1–1 phenotype into three subtypes 1F 1F 1F 1S and 1S–1S Chemical studies of α chains made by *Smithies et al* (5) and *Black & Dixon* (6) revealed that 1Fα and 1Sα differ only in one amino acid (lysine in Hp 1Fα is replaced by glutamine in Hp 1Sα) and that the polypeptide Hp 2α is essentially Hp 1Fα plus Hp 1Sα

The subtyping of haptoglobins by electrophoresis of purified haptoglobins in urea mercapto ethanol gels is laborious This reduces the practical usefulness of these characteristics as markers in genetic studies and forensic serology An immunological method for subtyping would be simple and should therefore make haptoglobin subtyping more useful

So far a distinction between Hp 1F–1F and Hp 1S–1S by serological methods has not succeeded However there are several instances where the products of allelic genes have been successfully differentiated using precipitating hetero antisera for example Ip (7) Gm (8) Gc (9) Hb (10) Therefore I made a further attempt to produce antisera specific for Hp 1F and Hp 1S which would allow Hp 1–1 sera to be subtyped by gel double diffusion

I purified 1F 1F and 1S–1S haptoglobins by the following method The sera were dialysed against 0 01 M Na acetate buffer pH 4 7 and applied to a column of DEAE Sephadex equilibrated with the same buffer The column was washed with 0 01 M Na acetate–0 03 M NaCl

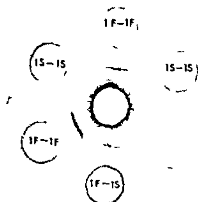


Fig 1

Double diffusion in agar gel The absorbed anti Hp 1F serum in the center tested against sera belonging to different haptoglobin subtypes

buffer and eluted with a buffer containing 0.01 M Na acetate 0.14 M NaCl The haptoglobin rich eluate was concentrated by ultrafiltration and dialysed against Tris (0.1 M) HCl buffer pH 8 Gelfiltration was performed in a 2.5 × 90 cm column of Sephadex G 200 (Pharmacia Sweden) with the same buffer and a flow rate of 6 ml/hour The fractions were tested immunologically by gel double diffusion with antisera against haptoglobin and normal human serum (anti NHS Behringwerke)

The fractions containing mainly haptoglobin were then dialysed against 1 per cent glycine and applied to an Electrofocusing column (LKB Sweden) 15 mg protein/run The column was run for 72 hours at 500 V in a pH gradient from 3 to 6 Fractions of 2.5 ml were collected and the protein content was determined by measuring the optical density The fractions were tested by gel double diffusion as before The fractions giving only one precipitin line against anti Hp and anti NHS antisera were pooled dialysed against phosphate buffer and stored at —20° C Sera were subtyped according to *Smithies* (3) Rabbits and sheep were immunized subcutaneously with 0.5 and 1 mg/injection respectively of the pure haptoglobin preparations emulsified in complete Freund's adjuvant (Difco) The injections were given weekly for one month and then every third week for at least 4 months The anti Hp sera obtained here called anti Hp 1F and anti Hp 1S usually contained minute amounts of antibody against other serum proteins These contaminating antibodies were removed by absorption with anhaptoglobinaemic serum (5 mg of lyophilized serum to 1 ml of antiserum) When several human sera belonging to different Hp groups were tested against the absorbed antisera no spur formation could be detected The anti Hp 1F serum was further absorbed with different amounts of 1S–1S serum and the anti Hp 1S similarly with

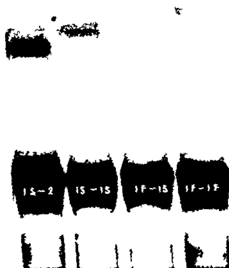


Fig 2

Starch gel electrophoresis patterns of some common haptoglobin subtypes

1F 1F serum Extensive absorption removed all reactivity against haptoglobin from the antisera but less thorough absorption resulted in an anti Hp 1F serum that reacted with Hp 1F–1F Hp 1F–1S Hp 1–2 and Hp 2 2 sera but not with Hp 1S–1S sera (Fig 1) The absorbed anti Hp 1S serum reacted with all but 1F–1F sera

So far I have analysed 20 1S–1S sera 10 1F–1F sera 20 1F–1S sera 15 1 2 sera and 60 2–2 sera and all the results agree with those obtained by subtyping by electrophoresis in urea mercapto ethanol gels (Fig 2)

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A PAPER DISC TECHNIQUE FOR STUDYING ANTIBACTERIAL SYNERGISM

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Received 4 xii 68

In routine sensitivity testing of bacteria by means of the paper disc method we occasionally observed that *Proteus* strains showed an inhibition zone of an irregular appearance at the colistin disc. This "wind screen wiper" zone the principal appearance of which is shown in Fig. 1 was only seen in the presence of a sulphonamide disc placed at a limited distance from the colistin disc. Our observation confirming some earlier reports of a positive interaction between sulphonamide and colistin (4) suggested to us a true synergistic effect of these drugs on *Proteus* and inspired a closer study.

Evidently paper disc methods can be used to study synergism. In fact such methods have been proposed (1–3). In a preliminary sensitivity test the inhibition zone diameters for the different antibiotics are recorded. A second plate is then inoculated with the same strain and two discs each containing one of the possibly synergistic drugs are placed on the agar surface at a distance from each other corresponding to half the sum of the previously observed zone diameters. If the antibacterial drugs have a synergistic action on the strain in question an increased effect is observed in the tangential region of the two inhibition zones.

A prerequisite for this method is obviously that at least one—and preferably both—of the drugs have some inhibitory effect on the strain. It has also been stated (1) that a drug must have some action alone against a micro organism in order to be an effective member of a synergistic pair. *Proteus* however is a species that is generally resistant to colistin (5) and often also to sulphonamides and consequently we had to modify the technique to suit even such conditions.

MATERIAL

The strains were isolated from clinical specimens sent to the laboratory for bacteriological diagnosis. In all 18 strains of *Proteus mirabilis* six of *Proteus vulgaris* and [illegible] of each of *Staphylococcus aureus* *E. coli* and *Enterococcus* were tested

Paper discs prepared as described by *Ericsson, Högman & Wickman* (2) were obtained from Karolinska Sjukhuset, Stockholm. The discs contained 2.4 mg of sulphonamide and 30 mcg of colistin respectively.

All tests were performed on agar plates prepared from Oxoid Blood Agar Base with the addition of 0.1 per cent glucose.

RESULTS

In the first part of the study we used the disc arrangement shown in Fig. 1. On an inoculated agar plate colistin discs were placed around a central sulphonamide disc at varying distances from the latter. The distances ranged from 7.5 to 40 mm. A gauge drawn on white paper and placed under the transparent plate was used to simplify the exact placing of the discs. The plates were left for two hours at room temperature for prediffusion and were then incubated at 37° C over night.

By means of this "spiral plate" method the 25 *Proteus* strains were investigated. All the strains had previously been found to be resistant to colistin and 12 of them also to sulphonamide. 24 of the strains showed a pattern similar to that in Fig. 1, while the remaining strain showed no evidence of synergism. At the colistin disc inhibition zones were observed varying in size and form with the distance between the sulphonamide and colistin discs. At a certain distance between the discs the typical "windscreen wiper" zone was seen. This distance differed for different strains. An imaginary line connecting the outer margins of the inhibition zones formed a "circle" with the sulphonamide disc as centre. When the distance between the discs was too large no inhibition zone was observed.

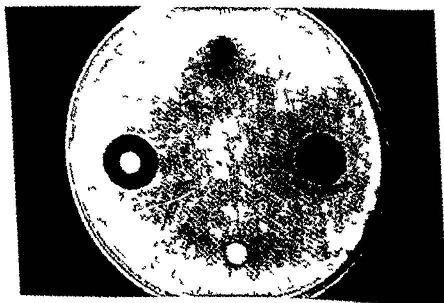
Experience and some preliminary experiments had shown that the antibacterial drugs in the paper discs diffused rather rapidly into the agar when the discs were placed on an agar plate. After one hour most of the drug appeared to be in the medium. Based upon this observation a "disc replacement technique" was developed. Two colistin and two sulphonamide discs were placed on an inoculated agar plate as shown in Fig. 2. To ensure rapid diffusion the discs were moistened with a loopful of sterile distilled water. The plate was left at room temperature for one hour. Then the sulphonamide discs were removed and replaced by a colistin disc and another sulphonamide disc respectively. The two original colistin discs were replaced by a sulphonamide disc and another colistin disc. The plate was left for two hours at room temperature for prediffusion and then incubated at 37° C over night.

By this technique the synergism between sulphonamide and colistin could be further established. Clear zones of inhibition were observed when a sulphonamide disc had been replaced by a colistin disc and *vice versa*. On the other hand no inhibition was obtained when the discs had been replaced by another disc with the same drug in spite of the doubling of the amount of the drug. The 12 *Proteus* strains resistant to both colistin and sulphonamide were tested with the "replacement tech-



Fig 1

A *Proteus m rab l s* strain tested by means of the spiral plate method (see text) In the centre a sulphonamide disc and ar und it at varying distances colistin discs



Fig

[illegible] ment technique ([illegible] text) applied to a *Proteus mirabilis* strain [illegible] A sulph namide di [illegible] replaced b an ther sulph namide disc (light A colistin [illegible] by a sulph namide di c Bott m A c listin disc replaced by another [illegible] A sulph namide disc repla ed by a colistin disc

Acta path microbiol scand 76 146–148 1969

BRIEF REPORTS

INTERFERENCE FILTERS FOR IMPROVED IMMUNOFLUORESCENCE MICROSCOPY

By *Jørgen Bygaard* and *Werner Olsen*

Exact calculation and production of interference filters with high transmission for use in fluorescence microscopy is possible to lay thanks to new design methods based on computer technique (5)

In this way a two band interference filter for use as a primary filter for tracing with fluorescein isothiocyanate (FITC) has been developed The main band used for excitation of the fluorochrome gives up to 85 per cent transmission in the 400–495 nm range (blue) With the light transmitted in this band a bright yellow green specific fluorescence can be obtained with an ordinary tungsten lamp (*e g* Zeiss low voltage 12 volt 60 watt) Autofluorescence is negligible using these wavelengths for excitation The second band gives about 1 per cent transmission in a narrow range around 630 nm (red) This band has been added for two purposes namely in order to obtain a highly contrasting background colour for the fluorescence and in order to allow easy localization of fluorescence For most purposes this red band will render any additional phase contrast equipment superfluous

The strongest activation of a fluorochrome is obtained by the wavelengths that are maximally absorbed by the dye (3) The most commonly used fluorescent conjugates are with FITC and have an absorption maximum at 495 nm in the visible part of the light spectrum with an additional medium high plateau around 300 nm in the invisible part of the spectrum The emission maximum of FITC conjugates is at 520 nm (3)

The optimal primary filter should have high transmission at the absorption maximum of the fluorochrome thus giving ample energy for excitation but must of course not overlap the emission maximum For practical purposes it has not so far been possible to apply this theoretical optimum of activation in tracing with FITC because the primary glass filters commonly available have not permitted satisfactory selective filtering The primary filter used in most studies published are the BG 12 filter from Schott & Gen Mainz or similar types of other brands often in combination with a red stopping filter *e g* type BG 38 This combination will give an ultraviolet blue transmission with its maximum at 400 nm As secondary filters are used yellow orange glass filters opening in the range of 500 nm *e g* OG 1 or OG 5 (2) The BG 12 primary filters are used with high pressure mercury lamps They will transmit only a small quantity of light at the theoretical optimum for activation of the fluorescence of FITC In addition being less effective activators of FITC the shorter light wavelengths may give rise to varying degrees of autofluorescence of tissues so that distinction between specific immunofluorescence and autofluorescence may be difficult and sometimes even impossible

The use of an iodine quartz illumination in combination with various filter combinations has been suggested (4 6) Tomlinson (6) sandwiched two Wratten gelatine filters Nos 32 and 38A giving 66 per cent transmission of 460 nm and 21 per cent transmission at 490 nm Good results were obtained but still the filter combination was characterized as being not ideal because of the low transmission at the wavelengths required for excitation

As pointed out by Nairn There is no doubt that performance of the fluorescence microscope can be improved if optimum filter systems are used (3)

Transmission curves of a new primary interference filter designed to meet opti

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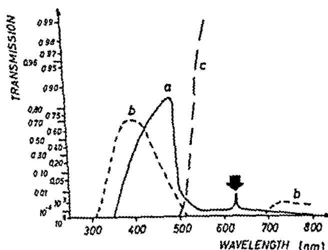


Fig. 1

Transmission curves of primary and secondary filters (a) Interference filter arrow indicating contrast band (b) BG 12/3 Schott & Gen (c) OG 1 Schott & Gen

mum requirements are given in Fig. 1. With FITC as the tracer a traditional set up for fluorescence microscopy has been compared with the new system in that primary filters BG 38 2.5 + BG 12/3 with the Osram HBO 200 as the light source were compared with the two band interference filter and the Zeiss 60 watt tungsten lamp. For both systems a Zeiss photomicroscope with the following equipment was employed: [illegible] condenser Zeiss dark field oil immersion; objective Zeiss Apo 40/1.0 oil immersion; optovar 1.25 ×; and eyepieces Zeiss Kpl 8 × giving a 400 × magnification. Secondary filters Zeiss 47, 50 and 53.

The results favoured the interference filter combined with the 60 watt lamp. The secondary filter of choice was the Zeiss 50 (515 nm) filter giving the most suitable red transmission of the three secondary filters tested and thus the best background contrast. Test objects were frozen sections of liver, thyroid, muscle and leucocyte smears tracing for antinuclear factors and other autoantibodies in human sera. A FITC conjugated anti human gamma globulin was used. Sections were mounted in buffered glycerol. In addition the fluorescent treponemal antibody (FTA) test was performed successfully with the interference filter system.

The main advantages of the interference filter are the bright specific immunofluorescence with a simple light source, the high contrast between fluorescence and background tissue and the easy localization of fluorescent areas in the cells or tissues. There is also negligible autofluorescence. Furthermore a so far unknown richness in details is seen due to both the absence of autofluorescence and the optimal colour contrast.

We believe that immunofluorescence with FITC conjugates will be facilitated by this new system and that new fields in searching for weak antigens and antibodies may be opened, e.g. in auto immunity and tumour immunity research. The system can be adapted for other fluorochromes also. The tungsten lamp may be replaced by a halogen lamp.

The light sources are stable thus facilitating microphotometry and need no heating period before use. The slightly higher cost of interference filters as compared to ordinary glass filters is more than compensated by the low cost of light source and transformer and the omittance of combined systems, e.g. phase fluorescence for orientation.

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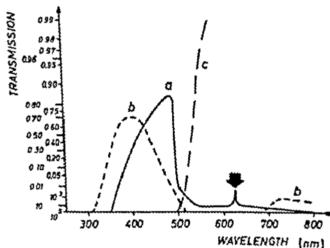


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We b l that immun flu rescence with FITC conjugates will be facilitated by thi n w s tem and that new fields in searching for weak antigens and antibodies may be pene ? g in auto immunity and tum ur immunity research The system an be adapted f other fluorochromes als The tungsten lamp may be replaced b a l al gen lamp

Th c light sources a e table thus facilitating micr photometry and need no heating peri d before use The slightly higher c st of interference filters as com ? t rdinary glass filter is m re than c mpensated by the low cost of light ur and transforme and the mittance of combined systems e g phase fluores n c f r r ntation

R f n s l (ons 4 H (e h H J & Jones R \ Proc Soc exp Biol (N Y)
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Acta path microbiol scand 76 148-149 1969

IDENTIFICATION OF AN ASACCHAROLYTIC *NEISSERIA* STRAIN CAUSING MENINGITIS

By *Kjell Bøvre*

Previous investigations revealed that quantitative genetic transformation can be a valuable tool for the correct arrangement of bacterial strains in a classification system (3) The following demonstrate the practical diagnostic application of this method in the identification of an aberrant *Neisseria* phenotype isolated from a patient with meningitis

Clinical Report

K K a boy born on January 1 1966 was admitted on August 1 1966 to the Pediatric Department Rikshospitalet University of Oslo Upon admission he had pronounced clinical signs of meningeal irritation and his cerebrospinal fluid contained numerous leucocytes Treatment was successfully performed with sulfonamide and penicillin G according to the bacteriological finding

Conventional Bacteriology

The strain B 8152/66 was isolated on a blood agar plate inoculated with cerebrospinal fluid from the patient on the day of admission It appeared as a homogeneous culture of dimethyl oxidase positive Gram negative diplococci with colonial characteristics typical of *Neisseria meningitidis* without pigment production or haemolysis The nitrate reduction test (in serum containing medium) was negative However all colonies examined were also negative in the ordinary tests for saccharolytic activities with 1 per cent glucose maltose and sucrose each incorporated in slants of nutrient agar with 24 per cent ascitic fluid The strain was highly sensitive to sulfonamide penicillin G streptomycin chloramphenicol oxytetracycline and erythromycin as revealed by a plate diffusion test (3)

On the basis of the characters mentioned these differential diagnoses were considered 1) asaccharolytic *N meningitidis* 2) nonpigmented *N flavescens* and 3) nitrate negative *N catarrhalis* The descendants of a single colony provided the material for the following investigation

Genetic Transformation

The wild type of strain B 8152/66 was used as recipient in transformation experiment which generally followed the quantitative procedure described (3) with stock transforming DNA preparations of streptomycin resistant (SR) mutants of *N catarrhalis* 1910/62 *N flavescens* ATCC 13115 and *N meningitidis* MF all of which had been employed previously (3) In order to provide a tentative species diagnosis in a few days autologous SR DNA had to be omitted at this stage The

Received 7 iii 69 from *Kaptein W Wilhelmsen og Frues Bakt r Iogiske Institutt University of Oslo and Rikshospitalet Oslo (Head Professor S D Henriksen)*

Credit is due to Mr *F Saltrøe* medical student who took part in the initial diagnostic procedure

numbers of streptomycin resistant transformants elicited by the three donors per ml of the same recipient population were as follows: *N. catarrhalis* 10 or less, *N. flavescens* 1 × 10, and *N. meningitidis* 1.6 × 10^3. These results strongly indicated the species diagnosis *N. meningitidis* for the new isolate. For confirmation another quantitative experiment was performed with identical aliquots of the recipient B 8159/66 simultaneously exposed to *N. meningitidis* M6 SR DNA and to the autologous B 8159/66 SR DNA. The interstrain reaction gave rise to 3.8 × 10^4 transformants per ml and the autologous reaction revealed 6.4 × 10^4 transformants per ml (0.01 per cent of the recipient count). The resulting ratio of inter strain to intra strain (autologous) transformation is in the order of 0.6, which is compatible with a first degree interrelationship as found between strains of the same species (3). The reciprocal quantitative experiment with *N. meningitidis* M6 as recipient was corroborative.

Further Cultural Tests

After having established the diagnosis by transformation, the saccharolytic potential was retested by daily readings for 10 days of cultures at 33 C and 37 C on the carbohydrate media described, and also on the same basis medium with 10 per cent of the respective carbohydrates. No positive reaction was observed except for one instance, where the 10 per cent maltose ascites agar slant revealed a weak acid reaction after 3 days at 37 C. Following 4 successive, probably nonselective subcultures from this slant on blood agar, it was finally observed that 9 out of 10 single colonies tested consisted of cells with the glucose+/maltose+/sucrose− pattern typical of *N. meningitidis*. Limited attempts to find a change to typical saccharolytic pattern also in other subculture lines of the same strain, including the streptomycin resistant mutant, all failed. The strain was also asaccharolytic on solid and fluid Mueller-Hinton media (2).

Comments

Glucose−/maltose−/sucrose− strains of *N. meningitidis* were described as early as at the beginning of the century (1). However, strain B 8159/66 is the first clinical isolate of this variety which has been shown to be *N. meningitidis* by genetic means. Recently Jyssum & Jyssum described mutator induced mutants of this species with a similar defect in carbohydrate metabolism (4).

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Acta path microbiol scand 76 150-160 1969

TRANSACTIONS
OF THE SWEDISH PATHOLOGICAL SOCIETY

Meeting November 29-30, 1968

Nilseth Ch. & Schnürer L. B. DISEASES OF THE THYROID IN AN AUTOPSY SERIES FROM MID SWEDEN

Strongly diverging figures on the frequency of primary thyroid malignant tumours in autopsy series have been published. In some large materials the figures were as low as 0.1 per cent but in more recent studies in which the thyroid was carefully examined figures as high as 1-3 per cent were reported.

Our material was obtained from 500 autopsies performed in 1968 at the Department of Pathology, Regionsjukhuset, Örebro. After weighing and fixation in formalin the thyroid was sliced and several slides including such from all grossly visible changes were studied using van Gieson as routine stain.

The average weight of the thyroid was found to be high, 32.4 g and it tendend to increase with age. High figures were also obtained for the frequency of goiter which was considered as present if the thyroid gland weighed 35 g or more.

Chronic lymphoid thyroiditis was found in 12, subacute thyroiditis in 2 cases.

Thirty two benign tumours were found.

Six primary malignant tumours, all carcinomas, were found, 4 occurred in men and 2 in women. Three were fairly well differentiated papillary carcinomas and 3 were small sclerosing rather papillary carcinomas. The frequency in our series 1.2 per cent corresponds fairly well to that of recent reports claiming that figures for primary thyroid malignant tumours in such specially studied series are 10 to 20 times higher than the figures obtained from routine materials.

Among 115 primary malignant tumours outside the thyroid 14 (12.2 per cent) had thyroid metastases, a surprisingly high figure considering the common statement that this condition is seldom encountered.

Enerbäck L. & Lundvall O. LIVER CELL FLUORESCENCE IN PORPHYRIA CUTANEA TARDA (PCT)

The liver of patients with cutaneous hepatic porphyria contains preformed porphyrins exhibiting red fluorescence after activation with ultraviolet or visible light. The properties and distribution of hepatic porphyrins were studied by fluorescence microscopy on aspiration biopsy smear and biopsy sections from 19 patients with latent or manifest PCT.

The porphyrins were found to occur in liver cell cytoplasm in freely diffusible form necessitating the use of special techniques for demonstration of the proper localization. Freeze drying and formaldehyde gas fixation or fixation in absolute ethanol followed by paraffin embedding gave good results provided that the sections were flattened in the dry state and mounted in water free media. If sections were floated onto water most of the fluorescent material was extracted from the sections and the remaining fluorescence was found in liver cell cytoplasm and nuclei or in portal connective tissue.

Air dried smears from fine needle aspiration biopsies contained red fluorescence of varying intensity localized to cytoplasm and nuclei of liver cells Various attempts to avoid the artefactive nuclear fluorescence were unsuccessful Liver cell fluorescence was found in all examined cases of PCT regardless of stage of clinical activity of the disease and in none of 30 control subjects without signs of the disease The extremely simple method of aspiration biopsy should be a useful diagnostic tool in cases of PCT

Hof r P A & Andersson R POST MORTEM FINDINGS IN A CASE OF FAMILIAL AMYLOIDOSIS WITH POLYNEUROPATHY

During the last few years several patients with primary familial amyloidosis with polyneuropathy have been observed in the Umeå hospital region comprising the Northern half of Sweden So far in 13 cases the diagnosis has been established by histological examination In 10 additional patients belonging in the same two families signs and symptoms suggestive of this disease have been observed.

The present case was a 5? year old woman showing progressive sensim tor peripheral neuropathy beginning in the lower extremities She also had malabsorption and vitreous opacities The diagnosis was established ante mortem by examination of biopsy specimens She died after about 6 years of illness at the age of 61

Post mortem examination revealed amyloidosis of peri-collagen distribution with deposits in the vessels of most tissues and organs In peripheral nerves there were important deposits of amyloid but nothing could be detected in the central nervous system Gross deposits were found in the *musculoris mucosae* and in the *tunica muscularis* of the gastrointestinal tract The opacities of the vitreous were shown histochemically to be amyloid Only slight deposits were found in the spleen and the liver The kidneys were moderately involved In the myocardium the amyloid was interstitially deposited Abundant deposits were found in the skin especially in relation to the sweat glands and in the arrector pili muscles.

The diagnosis of this type of amyloidosis is best established by biopsy of skin ctal mucosa and peripheral nerves.

Ukulom ki J & B rge Th ALVEOLAR RHABDOMYOSARCOMA

Three ca es f alveolar rhabdomy sarcoma were rep rted In one of the cases a w man 34 years ld the tum ur was located in the breast As far as we kn w this l ati n h n t been lescribed bef re An ther ca e was a 3 year-old b y with generaliz d gr wth f mixed embry nal and alveolar rhabdomyosarcoma The third ase wa a b y 2 years ol I who was well and with ut signs f tum ur 4 years after di gn s A detaile l de cription f the path l gy was given and the differential liagn s wa discu ed The finding were orrelated with literature cases

Publ in A ta path microbi l scand ? 782 790 1969)

Oll ng I GASTRIC ULCERA OF NEWBORN INFANTS

U l c a f the st mach f f ur new b rn infants (three boys and one girl) dying n natally are described The time of survival varied between 7 and 50 hrs and birth weight aried between 3? 0 and 3470 gm In two cases there was Rh in mpatibility with evere immunization of the children and exchange transfusions wer perf rmed soon after delivery In another case the mother got appendicitis n l wa perated n one day bef re delivery and the infant showed signs of severe

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The diagnosis of this type of amyloidosis is best established by biopsy of skin rectal mucosa and peripheral nerves

Mikulowski P & Berge Th ALVEOLAR RHABDOMYOSARCOMA

Three cases of alveolar rhabdomyosarcoma were reported In one of the cases a woman 34 years old the tumour was located in the breast As far as we know this location has not been described before Another case was a 3 year old boy with generalized growth of mixed embryonal and alveolar rhabdomyosarcoma The third case was a boy 2 years old who was well and without signs of tumour 4 years after diagnosis A detailed description of the pathology was given and the differential diagnosis was discussed The findings were correlated with literature cases

(Publ in Acta path microbiol scand 75 282-290 1969)

Olding L GASTRIC ULCERA OF NEWBORN INFANTS

Ulcera of the stomach of four newborn infants (three boys and one girl) dying neonatally are described The time of survival varied between 7 and 50 hrs and birth weights varied between 1670 and 3470 gm In two cases there was Rh incompatibility with severe immunization of the children and exchange transfusions were performed soon after delivery In another case the mother got appendicitis and was operated on one day before delivery and the infant showed signs of severe

immaturity *The fourth infant had a severe cardiac malformation* In all cases multiple gastric ulcera were found at autopsy In one case (of Rh incompatibility) *there was also perforation* peritonitis and septicaemia due to Klebsiella bacteria The other three cases showed no perforation and the autopsy cultures were negative *There was no or very little inflammation* around the ulcera except in the case of perforation Two types of ulcera were revealed In two cases extensive haemorrhages in the gastric wall and haemorrhagic necroses around the ulcers were found In the other two cases there was a curious homogenization of the connective tissue at the bottom of the ulceration and silver impregnation revealed extensive necroses of the reticular fibers probably due to peptic action of the gastric juice No notable haemorrhages were seen in these two cases.

Crant C A Erilsson B Holm A I Ivemarl B C Wallgren C THE ANATOMICAL FINDINGS IN SO CALLED HYPOPLASTIC LEFT HEART SYNDROME

Schnürer L B Fritjofsson A Lindgren A Magnusson P H & Pettersson S FINE NEEDLE VERSUS COARSE NEEDLE IN PUNCTION DIAGNOSIS OF PROSTATIC CARCINOMA

In 163 cases of suspected prostatic carcinoma fine needle punction transrectally according to *Fran én* and coarse needle punction through perineum according to Veenema was performed on the same occasion and the histological and cytological findings were compared

Histologically 85 cancers (52 per cent) were found and the frequency increased with age from 35 per cent (50-59 years) to 78 per cent (80 years and more) A complete agreement between histology and cytology was found in 87 per cent of the subjects if suspicious cases were included among the cancer cases the figure is 91 per cent Among 82 histologically proven carcinomas 76 were diagnosed cytologically and another 2 were suspected One case was cytologically negative and three smears were too scanty Later on three of the cytologically positive but histologically suspicious or negative cases were shown to have cancer

Considering the good agreement between results obtained by the two methods and the fact that fine needle technique is less inconvenient to the patient and gives fewer complications than the coarse needle method the authors recommend the fine needle aspiration technique in cases of clinically suspected carcinoma

Cranberg Ingrid PROSTATIC ASPIRATION BIOPSIES FROM PATIENTS WITH AND WITHOUT SYMPTOMS OR ABNORMAL PALPATORY FINDINGS FROM THE URINARY TRACT—A COMPARATIVE INVESTIGATION

Akerman M Brunk U Hallböl T & Stormby N C FINE NEEDLE BIOPSIES OF PANCREAS DURING OPERATION

Dahlgren S E & Ovenfors C O ASPIRATION BIOPSIES OF INTRATHORACIC NEUROGENIC TUMOURS

Schnürer L B & med kand Wittbolt S AN ADP SYSTEM FOR GYNAECOLOGICAL HEALTH CONTROL

Acta path microbiol scand
76 150–160 1969

than those in the lower part of the gradient as well as those in the pellet These enzymes included NADH and NADPH cytochrome c reductase NADH ferricyanide reductase and IDPase The highest specific activity of G 6 Pase was found in the fractions exhibiting a sedimentation velocity which was somewhat higher than that of the top fraction In contrast both the AMPase and Mg^{++} ATPase activities gradually increased from the upper to the lower part of the gradient and reached their maximum in the pellet These results strongly indicate the complex nature of the smooth microsomes and possible specialization of the functions within these membranes of the cytoplasm

Jakobsson S V COMPOSITION AND FUNCTION OF KIDNEY CORTEX MICROSOMES

The epithelial cells in kidney cortex have well developed plasma membranes as well as endoplasmic reticulum and it is therefore to be expected that the composition of the total microsomal fraction from the cortex should be complex The composition of the cortical microsomes was investigated by subjecting them to a subfractionating procedure After homogenization of the cortex in diluted tris buffer the membranous elements of the mitochondrial supernate were concentrated by centrifugation The suspension layered on a Ficoll gradient range 0–9 per cent in 10 per cent sucrose was centrifuged in a SW 25 rotor at 46 000 g for 60 min

10–15 per cent of the total microsomal protein was recovered in the pellet and 50–55 per cent remained in the upper part of the gradient The fractions exhibited a similar PLP/protein ratio but the cholesterol/PLP ratio was higher in the bottom part of the gradient For enzymic analysis the gradient was divided into pellet and four subfraction The membranes of the two fractions constituting the top of the gradient display on a protein basis high specific activities for the enzymes catalyzing the oxidation of NADH and NADPH—the former including the activities of flavoprotein NT reductase and cytochrome c reductase as well as the amount of cytochrome b and the latter measured as the activities of flavoprotein NT reductase enzymic lipid peroxidation and the amount of cytochrome P 450 These fractions also display an enrichment in IDPase activity The pellet and the fraction above it have shown a concentration (up to 80–90 per cent) of AMPase, Mg Na K ATPase and p NPPase G6Pase activity in general, corresponds to the distribution of electron transport enzymes but a minor part of the hydrolytic activity is also attributable to the membranes in the other fractions

It appears that the plasma membrane fragments of the microsomal fraction of kidney cortex exhibit a high concentration in the pellet on a Ficoll gradient In contrast the inner cytoplasmic membranes have a much broader sedimentation pattern situated with the highest specific activity close to the top of the gradient

Brunk U & Ericsson J L E FINE STRUCTURAL AGE CHANGES IN PONTINE NERVE CELLS

Nerve cells are suitable for studies of cellular age changes in mammals The age of the cells can be fairly accurately determined since they become differentiated early during embryonic development and apparently do not divide after birth

The present study was undertaken with the aim of studying the cellular alterations accompanying the accumulation of lipofuscin in ageing nerve cells with particular attention to the role of lysosomes and lysosomal enzymes Sprague

Acta path microbiol scand
76 150-160 1969

Dawley rats 3 weeks to 24 months old were anaesthetized with Nembutal and the brains were fixed by perfusion with a prewarmed (37 C) 1 5 per cent cacodyl ate buffered glutaraldehyde solution Acid phosphatase was demonstrated by in cubation of ~ 50 μ thick sections of glutaraldehyd fixed tissues in a modified Gomori medium

Electron microscopy of postosmicated thin sections revealed the occurrence of numerous lipofuscin like acid phosphatase positive cytoplasmic bodies in pontine nerve cells of old (19 to 24 months) rat In young (3 to 8 weeks) rats such bodies were few or absent cytoplasmic organelles carrying acid phosphatase (and pre sumed to represent lysosomes) were comparatively few and small and had a finely granular matrix containing occasional membranous profiles and/or homogeneous globular structures Cellular autophagocytosis was noted at all ages Apparent transitional stages between cytosegresomes (autophagic vacuoles) and lipofuscin bodies were often observed Acid phosphatase was demonstrated in fenestrated Golgi associated cisternae and also in coated" vesicles (primary lysosomes) which seemed to be budding out from these cisternae

The observations indicate (1) that lipofuscinlike bodies are lysosomes (residual bodies) (2) that these bodies are—at least in part—created through cellular autophagy (3) that there is a continuous new production of lysosomal enzymes throughout the life span of the cells and (4) that newly formed enzymes are enclosed in coated vesicles budding off from Golgi associated cisternae (and pre sumably represent primary lysosomes) The accumulation of lipofuscin granules may result from an inability of nerve cells to rid themselves of waste product accumulating in residual bodies

Sourander P *C Olsson Y* HISTOLOGICAL CHANGES OF THE SYMPATHETIC NERVOUS SYSTEM IN DIABETES MELLITUS

Pontén J *C Hugosson R* LONG TIME CULTURING OF NORMAL AND NEO PLASTIC HUMAN GLIA

Westermark B GROWTH CONTROL OF NORMAL AND MALIGNANT GLIA *IN VITRO*

Normal human glia cells in tissue culture are extremely sensitive to proximity depending cell cycle inhibition which means that the cell division stops when the cells have formed a confluent monolayer

The degree of cell cycle inhibition among normal and malignant glia cells has been studied in co cultivation experiments

In experiment one normal glia cells were seeded on top of a preformed stationary monolayer of glia cells As a control glia cells were seeded alone Cell count revealed that the top cells were completely inhibited by the bottom cells while the glia cells seeded alone proliferated logarithmically

In experiment two cells from the established glioma line 138 MG were seeded on top of a preformed monolayer of glia cells A control consisted of glioma cell alone The glioma cells proliferated logarithmically with the same rate when seeded alone or on glia cells

These experiments show that a confluent glia monolayer inhibit normal but not malignant glia cells Whether this reflects a qualitative or quantitative defect in the growth control of the neoplastic cells is not known

Acta path microbiol scand
76 150 160 1969

Lagerlöf P & Sundelin I ELECTRONMICROSCOPICAL CHARACTERIZATION OF HAEMOLYSIN PRODUCING CELLS IN MYELOID LEUKAEMIA IN FOWL

The viru induced myeloid leukaemia in fowl can be superimposed by anaemia f haemolytic type Some of the animals sh w transient positive Coombs test In order to elucidate whether these findings reflect an actual disturbance of the immuno log cal behaviour of the chicks we have tested the antibody producing capacity of various cells of the leukaemia chicl by use of the Jerne haemolytic plaque technique The cells from pl en and peripheral bl od of some of the leukaemic chi ks were shown to be as a tive antibody producers as those from non leukaemic chicks after antigenic stimulation with sheep red blood cells Electron micro scopical analysis showed that the plaque forming cells were plasma cells in all the non leukaemic case Similar cells were found in some of the plaque from leukaemic chicks In some cases the cells in the centre of the plaque were indi tinguishable ultrastructurally from myeloid leukaemia cells however The implications of these findings were dis ussed

Nilsson K LYMPHOBLASTOID TRANSFORMATION IN LONG TERM CULTURE OF HUMAN TISSUE

The term lymphoblastoid transformation (l tr) (*Beny sh M Inicl* 1963) de scribes the establishment *in vitro* of permanent lymph id cell lines from human lymph id tis ue Until 1967 l tr was considered to occur onl in cultures of malig nant lymphoma of leukaemic tis ue The incidence of l tr in different tissue culture systems has been reported to be very low Previously (Int J Cancer 3 183–190 1968) we reported the best results so far (50 per cent) obtained in cultures of normal and malignant lymph nodes We use a modified Trowell Jensen rgan culture and can now report l tr in 17 consecutive specimens from normal human lymphoid tissues Morphologically the established cell lines consist of blastoi l cells in the lymphoid series The cells depend on fibroblasts for their survival and preserve a highly differentiated function refle ted by a mon clonal production of IgG or IgA (l case) or IgM (1 case) The light chain is always kappa

Since infinite growth *in vitro* is considered a criterion of malignancy for somatic human tissue it is remarkable that permanent cell lines from human lymphoid tissue can be established in perhap 100 per cent To explain this finding three hypotheses are presented

A Normal lymphoid ti sue c ntains some cells capable of infinite proliferation

B Normal lymphoid tissue contains neoplastic cell clones controlled *in vivo* by unknown mechanisms

C Infinite growth of n rmal lymphoid cells is *ind iced in vitro* by unl nown agent (virus? antigen?)

Ulting I MATERNO FOETAL TRANSFER OF LYMPHOCYTES IN HUMAN SUBJECTS

Tran pla ental transfer f lymphocytes from mother to foetus was tudied by searching for cells with a female karyotyp e in the cord blood of newborn boys The leucoc te cultur technique of Moorhead et al (1960) w s employed. Only meta phases with 46 hr mos mes were included in the study Up to now eight newborn b s have been examined In two of them lymphocytes with a female karyotype ere dis overed In one case one cell out of 135 was female and in the other ase 2

cells out of 145 In the other six cases altogether 403 metaphases were analysed no female karyotype was found In these six cases however the total number of examined cells per individual varied between 29 and 177 because of varying quality of the chromosome preparations and it is possible that the frequency of trans placental cell transfer might have been larger than the one indicated by the result ing figures (2/8) if a larger number of cells could have been analysed The theo retical consequences of maternofoetal chimerism might be the production of immunoglobulins by maternal cells in the foetus or the foetal ability of delayed hypersensitivity reactions acquired from the mother

Hagmar B & Boeryd B STUDIES ON THE EFFECT OF HEPARIN ANTIPRO THROMBIN AND EPSILON AMINOCAPROIC ACID ON THE FORMATION OF SPONTANEOUS METASTASES

Anticoagulants and antifibrinolytics have been described to decrease and increase respectively the formation of metastases in allogeneic systems The effects of heparin antiprothrombin (phenprocoumon Marcoumar) and epsilon aminocaproic acid (EACA) were now tested on the formation of spontaneous metastases from resectable syngeneic tumours transplanted to the tails (20 methylcholantrene in duced rhabdomyosarcoma MCG 1 SS in CBA mice) The periods of treatment six days were varied in order to affect different phases of the metastatic process The metastasis dissemination started between day 7 and 13 after transplantation and treatment was first instituted between these days followed by resection of the tumours Heparin increased the average and total volumes of metastases to the lungs phenprocoumon decreased the number and total volume while EACA de creased the average and total volumes Resection on day 13 followed by treatment during 6 days revealed no differences between treated groups and controls In stitution of treatment on day 1 followed by resection on days 5 7 and 9 gave a higher incidence of pulmonary metastases only in heparin treated animals resected on day 5 Heparin increased the spontaneous metastasis formation while phen procoumon decreased it Heparin seems to promote an earlier release of tumour cells from the tumours EACA reduced the formation of spontaneous metastases

Norrby K Boeryd B Knutson F & Lundin I M EXPERIMENTAL STUDIES ON CIRCULATING TUMOUR CELLS

Factors influencing the pattern of secondary metastatic tumour growth are clinically as well as experimentally poorly understood In two murine syngeneic tumour host systems MCG 1 SS (solid) and AA (ascites) CBA and Melanoma B 16/C57Bl the number of monodispersed tumour cells required for progressive tumour growth after intravenous intraperitoneal and subcutaneous transplantation was determined In both systems about 10^3 more cells were required for successful intravenous transplantation compared to subcutaneous and intraperitoneal trans plantations

Intravenously transplanted ^{3}H thymidine labelled MCG 1 SS and AA tumour cells (label index about 85) were studied by radiochemical and autoradiographic techni ques Most of the injected cells were primarily trapped in the lungs However the DNA bound isotope activity in the lungs decreased quickly After 6 hours about 15 per cent of the injected activity remained in the lungs after 24–48 hours only a few per cent The activity decreased at the same rate in other tissues including the liver

The fact that considerably more cells are required for successful transplantation intravenously than subcutaneously and intraperitoneally is probably due to an extensive and rather quick disintegration of intravenously transplanted cells This disintegration of highly viable monodispersed syngeneic tumour cells in the circulation is thus far unexplained These matters are being additionally studied

Lindberg L G VIRUS PARTICLES IN ROUS SARCOMA OF HAMSTERS

Ericsson J L E GLUCAGON INDUCED CELLULAR AUTOPHAGY IN HEPATOCYTES

Intraperitoneal administration of crystalline glucagon to rats (50 µg/100 g of body weight) promptly induces greatly enhanced cellular autophagy in hepatocytes Previous studies of such cells containing prelabelled heterophagic secondary lysosomes (thorotrast or iron labelled) indicated that forming and early cytosegresomes (autophagic vacuoles) lacked lysosomal enzymes and acquired these enzymes by merger with pre existing lysosomes usually of secondary type

The present investigation was conducted in order to elicit the origin of the membrane(s) constituting the wall of early cytosegresomes The livers of glucagon *treated* rats were fixed by perfusion with 1 5 per cent buffered glutaraldehyde and 50 µ thick frozen sections were incubated in appropriate substrates for the fine structural demonstration of typical plasma membrane endoplasmic reticulum and Golgi enzymes These included adenosine triphosphatase (ATPase) for plasma membrane glucose 6 phosphatase (G 6 Pase) and inosinediphosphatase (IDPase) for endoplasmic reticulum and thiamine pyrophosphatase (TPPase) for Golgi membranes High resolution electron micrographs of thin sections of Vestopal embedded tissues were taken with special references to the ultrastructure of those membranes which appeared to be involved in the segregation of cytoplasmic organelles in glucagon treated animals

The findings indicated that the membranes surrounding forming cytosegresomes were derived from the endoplasmic reticulum since they showed activity of G 6 Pase and IDPase and were triple layered with a thickness of ~ 60 Å ATPase activity was not demonstrated in these membranes TPPase was present both in Golgi and endoplasmic reticulum membranes of control and experimental animals and in membranes participating in the sequestration of cytoplasmic organelles in animals given glucagon

Med kand Hansson H A & Sourander P ELECTRON MICROSCOPICAL INVESTIGATION OF LYSOSOMES IN TOXOPLASMA GONDII

Ljungqvist A THE INTRARENAL VESSEL ARCHITECTURE IN EXPERIMENTAL HYPERTENSION

Falkmer S B quist L & Hellman B SULPHHYDRYL GROUPS AND PANCREATIC ISLET TISSUE

In order to test the validity of the sulphhydryl (SH) theory for the pathogenesis of alloxan diabetes the following experiments were made

Assays of the glutathione (GSH) content in microdissected mammalian pan

creatic islets with 80 90 per cent β cells by a micromodification of the orthophthalaldehyde procedure showed that the CSH content of the islet parenchyma was higher (20 30 mg/100 g) than that of the acinar tissue (10–15 mg/kg) in all species studied (man rats AO mice)

Feeding non diabetic Chinese hamsters a diet deficient in cystein methionine did not increase the low frequency of spontaneous diabetes mellitus that occurs in this species but evolved a pathological glucose tolerance in 90 per cent of the animals Moreover in 2 hamsters (of 38) degranulation and necrosis were observed in some islets selectively affecting the β cells

Dithiol inhibitors as $CoCl_2$ $CdCl_2$ and $NaAsO_2$ were found to be able to evoke hyperglycaemia in the daddy sculpin and selective necroses in the dark central region of the principal islets where the β cells occur However no CSH lowering effect like that obtained after alloxan was observed

Excessive methionine administration (0 5 g/kg i p for 2 weeks) supposed to produce an SH compound imbalance was found to evoke atrophy of the exocrine pancreatic parenchyma in the Chinese hamster In 2 animals (of 42) selective β cell necrosis was observed in the islets

Though the CSH experiments did not conform to the SH theory the other observations indicate that SH compounds are of greater importance for the β cells than for the other islet parenchymal cells

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RETICULOENDOTHELIAL ACTIVITY RELATED TO AGE AND SEX IN MICE

By

F Hartveit and K Andersen*

Received 1 xi 68

In the course of experiments preliminary to work on the measurement of clearance time following the injection of carbon particles in mice (1) it became evident that female mice were able to clear such particles *quicker* than males of the same age This unpublished observation was followed up and the results are presented here

MATERIAL AND METHODS

Mice of the closed colony kept at this Institute were used The numbers used their age and body weight are shown in Fig 1

The carbon clearance time was determined after the intravenous injection of a suspension of Pelikan ink (C11/1431a) in physiological saline containing 1 per cent gelatine Blood samples were taken at timed intervals after injection and examined microscopically for carbon as described previously (1) the dose used being equivalent to 8 mg/100 g body weight

After determination of the clearance time the mice were killed and the liver and spleen removed and weighed

RESULTS

Clearance time The results are given in Table 1 which shows that at the age of 1 month the clearance time was similar in both sexes The clearance time then increased in both sexes with increasing age but this increase was much more marked in the males than in the females the sex difference being statistically significant at 3 and 6 months ($0.01 > P > 0.001$ and $0.001 > P$)

Organ weights Fig 2 shows that the liver weight increased with age The spleen weight was greater at 6 than at 1 month but showed great variation at 3 months (Fig 3) In both cases the male values tended to be greater than the female values but significant differences were not found The relative organ weight was similar in both sexes and tended to decrease with age (Table 2)

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TABLE 1

Mean Carbon Clearance Time (± S D) Related to Age and Sex in Mice

Age (months)	Clearance time (mins)	
	Male	Female
1	18 2 ± 7	19 1 ± 6
3	39 3 ± 6	28 6 ± 3
4	57 4 ± 8	33 2 ± 6

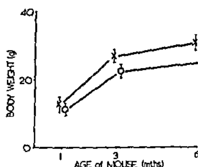
For number of mice see Fig 1

TABLE 2

Relative Organ Weight Related to Age in Mice

Age (months)	Organ weight (mg/g body weight)			
	Liver		Spleen	
	male	female	male	female
1	[illegible]	61	4	5
3	[illegible]	[illegible]	3	2
6	48	50	3	3

For number of mice see Fig 1



No of MICE			
♂	10	6	5
♀	10	6	6

Fig 1

Body weight related to age and sex in the mice used in the present experiments Male values X female 0 ± S D

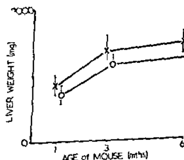


Fig 2

Liver weight related to age and sex in the mice used in the present experiments (For number of mice see Fig 1) Male values X female 0 ± S D

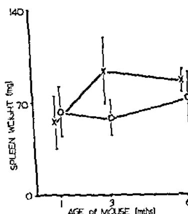


Fig 3
Sple n weight related to age and sex in the mice usel in the present experiments (For number of mice see Fig 1) Male values X fem le 0 ± S D

DISCUSSION

Reticuloendothelial activity is commonly measured by determination of the time required for the removal of inert particles from the blood stream Such particles are taken up by phagocytic cells in all parts of the body—but the majority are cleared from the circulation by the kupffer cells of the liver and their counterparts in the spleen As the size of these two organs and their relationship to total body weight changes with age it is reasonable to expect this to influence the clear ance time when dosage is given according to total body weight as in the present experiment The increase in clearance time with increasing age in both sexes can probably be in part explained in this way The sex difference in clearance time can however not be accounted for on this bases as the relative organ weights were remarkably similar in both sexes The difference appeared only after the mice had reached maturity This coupled with the finding that oestrogens may cause an increase in phagocytic activity in male mice (2) makes it likely that the differ ences observed in the present experiment are the result of hormonal action

SUMMARY

The clearance time for carbon particles in mice increases with age and is less in adult female than male mice The latter is probably due to the stimulating effect of oestrogens on phagocytosis

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SALICYLATE-INDUCED FOETAL HAEMORRHAGE IN TWO MOUSE STRAINS

By

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Received 15 xi 68

In teratological experiments with salicylates a high rate of foetal resorption has been found (*Warkany & Takacs* 1959 *Goldman & Yakovac* 1963 *Klein Obbink & Dalderup* 1964 *Larsson & Bostrom* 1965) An interesting observation in earlier experiments on mice was the increasing incidence of foetal mortality the later in pregnancy salicylate was administered to the mother (*Larsson & Eriksson* 1966) The possible mechanisms underlying the salicylate induced prenatal mortality were discussed Haemorrhage has been suggested to cause foetal death when acetylsalicylic acid has been administered to pregnant rats (*Brown & West* 1964)

In further investigations in our laboratory with special reference to drug induced late foetal death a high incidence of superficial haemorrhage was found in the foetus when salicylate was given to the mother on the 16th or 17th gestation day Moreover a special form of sub capsular haemorrhage in the liver attracted our attention when the abdomen of the foetus was opened for inspection of the viscera

The present study was undertaken for further investigation of these observations and of their possible relation to foetal mortality

It also seemed of interest to compare in this respect two mouse strains with a known difference in their susceptibility to salicylate (*Larsson & Bostrom* 1965)

MATERIALS AND METHODS

A totally of 64 pregnant primiparous mice of the A/Jax strain and 52 of the CBA strain were used They were mated overnight and on the following morning vaginal plug could easily be observed in most cases (*Larsson* 1962) This day was denoted as zero day of pregnancy

I take this opportunity of expressing my sincere gratitude to Dr K Sune Larsson for valuable advice during preparation of the manuscript I also wish to thank Mr *Lennart Nilsson* for photographical assistance and Mrs *Tollie Palm* and Miss *Eva Löfgren* for skilful secretarial and technical assistance

The expenses of the investigation were defrayed by grants from Reservations anslaget for framjande av medicinsk forskning from the Swedish Medical Research Council (14X-993-02 and 14P-993-02) and from grants to Drs K S Larsson and H Boström from the Association for the Aid of Crippled Children N Y U.S.A

TABLE 1

Effect of Sodium Salicylate on Foetal Mortality, Superficial and Liver Haemorrhages in the Foetuses, the Drug being Administered to the Mother in a Single Dose of 10 mg/20 g on Gestation Day 16, 17 or 18

Strain	Gestation day injected	Time of sacrificing after inj hrs	No of litters	No of foetuses	Foetal mortality		Superficial haemorrhage in living foetuses		Liver haemorrhage in living foetuses	
					no	%	no	%	no	%
A/Jax	16	8	7	48	22	46	11	42	5	19
CBA	16	8	5	39	1	3	18	49	4	11
A/Jax	16	24	7	46	20	43	15	56	7	26
CBA	16	24	8	46	3	7	9	21	0	0
A/Jax	17	2	6	36	0	0	1	3	1	3
A/Jax	17	4	6	40	0	0	14	35	1	3
CBA	17	4	6	36	0	0	12	33	1	3
A/Jax	17	8	7	47	9	19	8	21	6	16
CBA	17	8	7	38	2	5	20	56		6
A/Jax	17	12	7	50	27	54	12	52	7	30
CBA	17	12	7	37	9	24	10	30	0	0
A/Jax	17	24	10b	49	19	39	6	20	6	20
CBA	17	24	9	31	4	13	2	7	0	0
A/Jax	18	8		51	0	0	0	0	1	2
CBA	18	8	5	33	0	0	0	0	0	0
A/Jax	18	24	7d	—	—	—	—	—	—	
CBA	18	24	5	—	—	—	—	—	—	—

a 1 delivered before dissection b 2 delivered before dissection c 3 delivered before dissection d All 7 delivered before dissection All 5 delivered before dissection. Newborns were not examined and are therefore not included

Sodium salicylate (10 mg/[illegible]0 g body weight in 0.1 ml of distilled water) was given i.m. in a single dose at 10 a.m. on gestation day 16, 17 or 18. The mice injected on the 16th and 18th days were sacrificed 8 or 24 hours after treatment and the foetuses were removed. The mice injected on the 17th day were sacrificed after 2, 4, 8, 12 or 24 hours (Table 1). If the female delivered before sacrificing this was recorded and the newborns were not examined.

The foetuses were divided into dead and alive. Early foetal resorptions which had evidently occurred before the mother had received the injection were not recorded.

Superficial haemorrhage and its exact site were noted. The abdomen was opened and the liver was inspected in situ for haemorrhage. The involvement of different lobes by the haemorrhage was recorded. Foetuses found dead in the groups sacrificed 24 hours after injection were, however, too severely macerated to be properly examined. The whole foetus was immersed in Bouin's fluid and on the following day the liver was removed and once more examined.—The mother's liver was also inspected before and after fixation.

The livers of at least two foetuses from most litters were taken for paraffin embedding, sectioning at 7 μ and staining with haematoxylin and eosin.

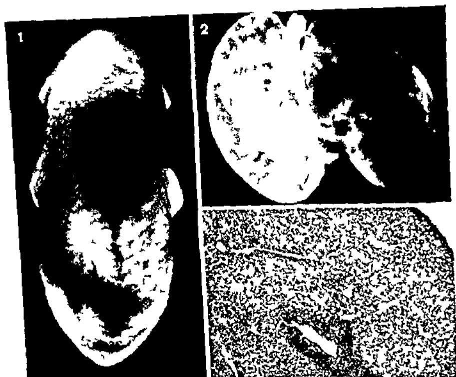
RESULTS

Foetal Death

The incidence of foetal mortality is expressed as the ratio of foetuses dead late in pregnancy to the total number of foetuses living and later dead (Table 1). In the A/Jax strain the maximum mortality of about 50 per cent was reached already 8 hours after injections given on the 16th day. If given on the 17th day foetal death of 19 per cent was found first 8 hours after injection increasing to a maximum of 54 per cent after 12 hours. In the CBA strain foetal death was seldom noted if injection was given on the 16th day, i.e. at most 7 per cent. If injections were given on the 17th day the incidence of foetal death was lower than that in the A/Jax strain, the maximum of 24 per cent being reached also 12 hours after injection. After injection on the 18th day no dead foetuses were observed in either strain. A tendency to premature delivery within 24 hours of injection was generally found after administration on days 17 and 18.

Superficial Haemorrhage

The incidence of superficial haemorrhage is expressed as the ratio of living foetuses with superficial haemorrhage to the total number of live foetuses (Table 1). The haemorrhage observed was situated along the spine (Fig. 1). In the A/Jax strain superficial haemorrhage was found if the drug was administered on the 16th day, increasing from 42 per cent after 8 hours to a maximum of 56 per cent after 24 hours. If administered on the 17th day the incidence was 35 per cent after 4 hours, increasing to a maximum of 56 per cent after 12 hours. In the CBA strain superficial haemorrhage was noted at almost the same rate as in the A/Jax strain. If injections were given on the 16th day, however, the maximum of 49 per cent was already reached at 8 hours with a decrease to 21 per cent at 24 hours. If given on the 17th day, as well, the maximum incidence of 56 per cent was reached after 8 hours



Figs 1–3

Fig 1 Superficial haemorrhage along the spine in a 17 day old A/Jax mouse foetus The mother was given sodium salicylate i m (10 mg/20 g) 8 hrs prior to sacrifice

Fig 2 Varying degrees of liver haemorrhage in a 17 day old A/Jax mouse foetus The mother was given sodium salicylate i m (10 mg/20 g) 8 hrs prior to sacrifice

Fig 3 HTX and eosin stained 7 μ section from the liver of a 17 day old A/Jax mouse foetus The mother was given sodium salicylate i m (10 mg/20 g) 12 hrs prior to sacrifice Normal liver cells are seen around the vessels Haemorrhages are visible subcapsularly × 60

All dead foetuses that could be examined had superficial haemorrhage

After injection on the 18th day no superficial haemorrhage was observed in either strain

Liver Haemorrhage

The incidence of liver haemorrhage is expressed as the ratio of living foetuses with such haemorrhage to the total number of live foetuses (Table 1)

The liver haemorrhage was seen subcapsularly and macroscopically did not extend into the parenchyma (Fig 2) Histological examination revealed necrosis and haemorrhage extending into the parenchyma

from beneath the capsule (Fig. 3) In a few cases focal necrosis was also present in microscopically unaffected foetuses

In the A/Jax strain liver haemorrhage was *found in approximately half of the living foetuses* with superficial haemorrhage on the 16th day On the 17th day only one foetus (3 per cent) had liver haemorrhage after 4 hours the incidence increasing to 30 per cent after 12 hours In the CBA strain on the other hand liver *haemorrhage was only occasionally found* on both days the maximum incidence being 11 per cent 8 hours after injection on the 16th day All dead foetuses that could be examined—except one 16 day old CBA foetus and two 17 day old CBA foetuses taken 8 hours after injection—had liver haemorrhage generally involving all lobes

After injection on the 18th day liver haemorrhage and histologically demonstrable necrosis were present in only one A/Jax foetus

DISCUSSION

The main question in this investigation is obviously the possible relation between the haemorrhages and the high foetal mortality Such a relation is indicated by the observation that a high percentage of foetal haemorrhage precedes the increased incidence of foetal death Moreover the fact that all dead foetuses which could be examined showed superficial haemorrhage and all but 3 CBA foetuses liver haemorrhage supports the view that haemorrhages are involved in the foetal death There were however almost always living foetuses with haemorrhage Whether or not these would have died later or whether their damage was less severe and might have been overcome can only be speculated

In view of the fact that the maximum mortality on the 17th day was as high as 54 per cent in the A/Jax strain– although death occurred somewhat later than on the 16th day—it is interesting to note that foetuses exposed to the drug on the 18th day were only rarely affected by the salicylate This difference in the susceptibility to salicylate may perhaps reflect the maturation of the foetus own detoxification enzyme system Drugs administered to the mother have been shown to affect the foetal liver enzyme activity (*Gordon et al* 1961 *Ordy et al* 1966) or to cause necrosis of the liver and delay its maturation (*Knoche & Vilmar* 1963) *This could presumably* also occur in the salicylate affected foetal liver with haemorrhage and necrosis

It is also interesting to observe that even if the CBA foetuses had superficial haemorrhage to almost the same extent as the A/Jax foetuses most of them seemed to survive The mechanism of this strain difference is not known

Another interesting feature is the presence of haemorrhage in the foetus but never in the mother The haemorrhage *seems to resemble the* petechial haemorrhages which have been observed in salicylate poison

ing *i e* it is present subcutaneously below the serous membranes and in a variety of organs (*Smith & Smith* 1966) Drugs such as quinine thiazide and coumarin have been reported to cause haemorrhage in the foetus or newborn when administered to the mother (*Posner* 1937 *Rodriguez* 1964 *Mer & Breitner* 1956) Salicylates and phenobarbital in excess have been stated to cause neonatal haemorrhage (*Apgar* 1964)

SUMMARY

Sodium salicylate in a single dose of 10 mg/20 g was given to pregnant mice of the A/Jax and CBA strains on gestation days 16 17 or 18 and they were sacrificed at various intervals after injection

Foetal death occurred after injection on the 16th and 17th day In the A/Jax strain the maximum mortality of about 50 per cent was reached earlier if injections were given on the 16th day (after 8 hours) than if they were given on the 17th day (after 12 hours) The incidence of foetal death was markedly less in the CBA foetuses

Superficial haemorrhage was observed in all dead foetuses and in about one half of the living ones after injection on the 16th and 17th day This applied to both strains

In the A/Jax strain liver haemorrhage with histologically demonstrable necrosis was present in all dead foetuses and in about one half of the living ones with superficial haemorrhage This was rare in the CBA strain

After injection on the 18th day foetuses seem only rarely to be affected and only those in the A/Jax strain

The possible relation between foetal mortality and induced haemorrhage is discussed

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MULTIPLE PRIMARY MALIGNANT TUMOURS

An Autopsy Study of a Circumscribed Population

By

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Received 25 xi 68

the true number of multiple cases can be estimated only by following the patients up as carefully as possible to the end of their lives and by examining all of them post mortem (Malmio 1959)

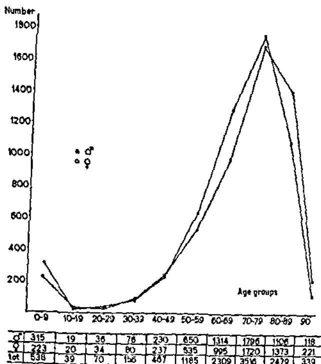
This is of course the ideal method but unfortunately very difficult to carry out Another possible way is to make use of an autopsy material and by means of the clinical records and the files of the department of pathology study malignancies earlier diagnosed in these subjects This is the method applied in this work and since we have been able to perform autopsy on about 60 per cent of those in a well defined population who died during the nine years 1958–1966 we think it worth while to describe the frequency of multiple cancer in these subjects

There are however many problems associated with a study of this type A patient with a slowly growing tumour like most of the prostatic carcinomas runs a greater risk of developing another cancer than for instance those with a malignant melanoma The results ultimately depend on diagnostic and therapeutic possibilities which may explain the different frequencies reported in clinical and autopsy series

In a later publication we will describe in detail some of the more frequent tumours where it is possible to evaluate the morbidity risk at different ages the frequencies of correct clinical diagnosis and the chances of survival It should then be possible to compare the expected frequencies of multiple tumours with those found

MATERIAL AND METHODS

Malmo is a town situated in the south of Sweden In 1966 it had about 250 000 inhabitants The town offers unusually good possibilities for medico demographic studies because it has a well defined population and as far as the medical service is concerned is a seperate region The town has only one general hospital (Malmo General Hospital) one infirmary for chronic diseases one hospital for mental diseases and only one department of pathology serving the entire area Out of the 18 675 who died during these years 11 098 where autopsied, i e 59.4 per cent The age and sex distribution of the material is given in Fig 1 The autopsies were performed according to uniform principles which are described in some detail earlier (Berge 1967)



	0-9	10-19	20-29	30-39	40-49	50-59	60-69	70-79	80-89	90
♂	315	19	36	76	230	650	1314	1796	1106	118
♀	223	20	34	80	237	535	995	1720	1373	221
Tot	538	39	70	156	467	1185	2309	3516	2479	339

Fig 1

Age and sex distribution of the 11 098 subjects autopsied 1958–1966

Data concerning previous treatment for cancer were taken from the clinical records which always were accessible before autopsy was performed Further the archives of the Department of Pathology were analysed in order to see if the patient earlier had been treated for cancer

DEFINITIONS

1 The term multiple tumours was used to designate two or more synchronous or metachronous tumours appearing in different organs The terms synchronous and metachronous were related to the situation at autopsy
2 The tumours were histologically malignant
3 The tumours were not metastases
4 One or more lesions with the same histological appearance (with unequivocal signs of malignancy) in any one organ or paired organs as well as primary generalized tumours (*e g* malignant lymphoma) were said to be one tumour
5 Latent prostatic cancer was included
6 Basal cell cancer was not accepted because of the biological benignity of this tumour and because data on previous treatment often were incomplete or missing
7 The group called *glioma* consists of different types of astrocytoma oligodendroglioma medulloblastoma and ependymoma
8 The term cured in cases treated for cancer is used in the sense *that recurrence* or metastases could not be demonstrated at autopsy
The data were processed with the computer in Lund

Thanks go to fil kand *Arne Sundstrom* Datacentralen Lund for most *valuable* assistance

TABLE 1

Age and Sex Distribution

Age group	Total	Subjects with 1 tumour			Subjects with 2 tumours				Subjects with 3 tumours				Subjects with 4 tumours				Subjects with multiple tumours in per cent of all
		Male	Female	Total	Male	Female	Total	Per cent of all subjects with tumours	Male	Female	Total	Per cent of all subjects with tumours	Male	Female	Total	Per cent of all subjects with tumours	
0–9	16	8	8	16	0	0	0	0	0	0	0	0	0	0	0	0	0
10–19	15	8	9	15	0	0	0	0	0	0	0	0	0	0	0	0	0
20–29	38	24	12	36	0	0	0	0	0	0	0	0	0	0	0	0	0
[illegible]	80	31	46	77	1	2	3	3.8	0	0	0	0	0	0	0	0	3.8
[illegible]	211	85	159	44	3	4	7	3.8	0	0	0	0	0	0	0	0	2.8
[illegible]	[illegible]	[illegible]	317	595	17	30	47	7.2	3	3	[illegible]	0.9	0	1	1	0.2	8.3
[illegible]	[illegible]	[illegible]	487	[illegible]	79	[illegible]	115	9.8	7	1	8	0.7	0	0	0	0	10.5
[illegible]	[illegible]	[illegible]	[illegible]	1727	117	55	19	1.5	12	2	14	0.9	0	1	1	0.07	13.5
[illegible]	[illegible]	[illegible]	114	[illegible]	[illegible]	41	175	11.1	10	8	16	1.5	1	1	2	0.2	14.8
[illegible]	[illegible]	[illegible]	[illegible]	85	[illegible]	[illegible]	22	20.0	1	1	2	1.8	1	0	1	0.9	22.7
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	10.6	11	13	18	0.9	2	7	5	0.1	11.7

RESULTS

Among the 11 098 autopsied subjects 4 895 (44.1 per cent) had or had had 5 523 primary malignant tumours.

The subjects with single and multiple tumours are given according to age and sex in Table 1.

It is seen from Table 1 that a single malignant tumour occurred in 4 323 subjects and multiple malignant tumours in 572, *i.e.* 11.7 *per cent* (521, 10.6 per cent had 2, 46, 0.9 per cent had 3 and 5, 0.1 per cent *had 4 tumours.*) Tumour was present at autopsy in 4 062 of the subjects with a single tumour and in 561 of those with multiple tumours. Among the 521 subjects with double cancers 377 had both tumours, 133 had 1 and 11 had none at autopsy. Among those with triple cancers, 25 had 3, 15 had 2 and 6 subjects had 1, and among those with quadruple cancers 1 had 4 and 4 had 3 tumours at autopsy. (More than 2 cancers had not been cured in any subject.) Multiple tumours synchronous at autopsy were thus found in 422 subjects while the tumours were metachronous at autopsy in 150 subjects.

TABLE 2

Number of Tumours at Different Sites and Types and Frequency of Syn- and or Metachronous Tumours

Site	Number	M. age at death	Frequency of multiple tumours: 2 tumours	3 tumours	4 tumours	Total	%
Carcinoma							
Prostate	808	76.5	215	26	2	243	30.1
Colon	482	7[illegible].6	92	12	2	106	22.0
Breast	478	67.4	79	9	1	90	18.8
Lung	475	69.0	83	5	1	89	18.5
Stomach	463	71.5	73	13	1	87	18.8
Kidney	251	70.4	70	13	0	83	33.1
Rectum	199	70.3	40	4	0	44	22.1
Pancreas	196	67.3	38	5	0	43	21.9
Liver	186	70.8	28	5	0	33	17.7
Ovary	173	63.9	17	1	1	19	11.0
Biliary system	172	72.2	23	1	1	25	14.5
Urinary bladder + urethra	149	72.6	23	3	1	27	18.1
Uterine cervix	129	60.8	15	3	0	18	14.0
Small intestine	113	[illegible]2.3	41	3	0	44	38.9
Uterine body	81	71.6	21	1	2	24	29.6
Oesophagus	78	71	19	0	0	19	24.4
Skin	69	65.8	9	6	1	16	23.2
Thyroid	54	63.8	11	3	1	15	27.8
Unknown	43	66.7	6	0	0	6	14.0
Renal pelvis + ureter	26	69.8	7	0	3	10	38.5
Larynx	21	69.4	5	2	0	7	33.3
Testis	16	43.4	2	0	0	2	12.5
Vulva	15	71.5	1	1	0	2	13.3
Appendix	14	45.1	2	3	0	5	35.7

TABLE 2 (cont.)

Site	Number	Mean age at death	Frequency of multiple tumours: 2 tumours	3 tumours	4 tumours	Total	%
Oral cavity	13	70.2	4	0	0	4	30.8
Anus	11	67.6	2	0	0	2	18.2
Uterus unspec	10	67.5	2	0	0	2	20.0
Eye	10	69.9	2	1	0	3	30.0
Maxillary sinus	10	75.0	2	0	0	2	20.0
Hypopharynx	9	70.8	1	0	0	1	11.1
Tongue	8	70.9	1	0	0	1	12.5
Lip	8	74.6	3	1	0	4	50.0
Salivary gland	8	73.1	1	0	0	1	12.5
Epipharynx	6	6[illegible].3	0	0	0	0	–
Penis	6	73.5	3	0	1	4	66.7
Adrenal	4	67.5	1	0	0	1	25.0
Vagina	3	80.3	0	0	0	0	–
Tube	3	63.3	0	0	0	0	–
Tonsil	2	66.5	0	0	0	0	–
Sweat gland	1	68.0	1	0	0	1	–
Trachea	1	65.0	1	0	0	1	–
Cardia	1	81.0	0	0	0	0	–
Other tumours							
Leucosis	217	60.5	34	6	0	40	18.4
Glioma	118	59.1	6	2	0	8	6.8
Reticulum cell sarcoma	113	67.3	21	3	0	24	21.2
Myelomatosis	76	7[illegible].4	11	1	0	12	15.8
Undiff. mesenchymal tumour	46	61.1	11	0	0	11	2[illegible].9
Hodgkin	4[illegible]	54.2	5	2	0		16.7
Mesothelioma	32	70.3	2	0	0	2	6.3
Leiomyosarcoma	24	73.4	4	2	0	6	25.0
Lymphosarcoma	12	69.8	2	0	0	2	16.7
Thymoma	8	64.4	1	0	0	1	12.5
Liposarcoma	6	78.5	1	0	0	1	16.7
Osteogenic sarcoma	5	37.6	0	0	0	0	–
Meningioma	4	75.5	1	0	0	1	25.0
Ewing sarcoma	4	16.8	0	0	0	0	–
Rhabdomyosarcoma	3	49.7	0	1	0	1	33.3
Chordoma	3	76.0	1	0	0	1	33.3
Brill Symmers	2	63.0	0	0	0	0	–
Mycosis fungoides	2	71.5	1	0	0	1	50.0
Neuroblastoma	1	62.0	0	[illegible]	0	0	–
Total	5593	69.7	1042	138	20	1200	

Treatment for one cancer had been successful in 413 subjects but in 152 of these (36.8 per cent) one or more other tumours were found at autopsy. Six (35.3 per cent) out of the 17 patients cured for 2 tumours were found to have another tumour at autopsy. The frequency of a second tumour increased with time after successful treatment but these problems will be discussed in detail in a later publication.

The series consisted mainly of aged subjects. The average age at death was 68.5 years in those with single cancers, 73.7 years in those

TABLE 3

Number and Locations (Types) of Multiple Tumours

Site	Number	Prostate	Colon	Stomach	Malignant lymphoma	Breast	Kidney	Lung	Uterus	Pancreas	Rectum	Small intestine	Liver	Urinary bladder + urethra	Biliary system	Skin	Ovary	Thyroid	Oesophagus	Renal pelvis — ureter	Larynx	Glioma	Appendix	Penis	Unknown site	All other tumours	Total
Prostate	808	–	33	30	20	0	29	37	–	20	15	13	18	11	6	5	–	3	7	2	3	2	0	3	1	15	273
Colon	482	33	–	13	10	11	4	5	6	4	1	8	3	6	3	0	1	5	0	3	0	0	1	0	0	3	122
Breast	478	0	13	13	9	[illegible]	8	6	12	5	3	2	0	2	2	4	9	1	0	0	0	0	0	–	0	9	100
Malign lymph	478	20	10	5	12	9	[illegible]	4	5	4	4	3	2	1	1	3	[illegible]	1	2	0	0	1	2	0	2	0	99
Lungs	475	37	5	6	4	6	4	2	4	1	3	[illegible]	4	2	3	0	0	0	2	0	2	0	0	0	0	4	95
Stomach	463	30	13	–	5	13	9	6	5	2	2	4	[illegible]	1	2	1	1	0	0	1	0	0	1	0	1	3	102
Kidney	251	29	4	9	6	8	–	4	1	4	2	1	5	1	2	[illegible]	3	3	2	0	2	1	0	0	0	3	96
Uterus	220	–	[illegible]	5	5	12	1	4	2	2	3	1	1	1	0	0	1	2	1	1	0	1	2	–	0	1	52
Rectum	199	15	1	2	4	3	2	3	3	1	–	1	2	1	4	1	0	1	0	0	1	0	0	0	0	3	48
Pancreas	196	20	4	[illegible]	4	[illegible]	4	1	[illegible]	–	1	3	0	1	0	0	0	0	0	0	1	0	0	0	0	0	48
Liver	18[illegible]	18	3	[illegible]	2	0	5	4	1	0	[illegible]	0	–	0	0	0	0	0	0	0	0	0	0	0	0	1	38
Ovary	173	–	1	1	2	9	3	0	1	0	0	0	0	1	0	0	–	1	0	2	0	1	0	–	0	0	22
Bil system	172	6	3	2	1	2	[illegible]	3	0	0	4	0	0	[illegible]	–	0	0	1	0	0	0	1	0	0	0	1	28
Urinary bladder + urethra	149	11	[illegible]	1	1	2	1	2	1	1	1	0	0	–	2	2	1	0	0	3	0	0	0	1	0	0	3[illegible]
Glioma	118		0	0	1	0	1	0	1	0	0	1	0	0	1	0	1	1	0	0	0	–	0	0	0	0	9
Small intest	113	13	8	4	3	[illegible]	1	6	1	3	1	–	0	0	0	0	0	0	1	1	0	1	1	0	0	1	47
Oesophagus	8	7	0	0	[illegible]	0		–	1	0	0	1	0	0	0	0	0	0	–	0	0	0	0	1	0	3	19
Skin	69	5	0	1		4	6	0	0	0	1	0	0	2	0	–	0	0	0	0	0	0	0	1	1	0	24
Thyroid	54	3	5	0	1	1	3	0	2	0	1	0	0	0	1	0	1	–	0	1	0	1	0	0	0	0	20
Total		249	115	96	95	91	91	89	49	47	44	44	37	[illegible]	27	22	20	19	15	14	9	9	7	6	[illegible]	47	1278

In malignant lymphoma are included also leucosis and multiple myeloma

with double cancers 73 8 years in those with triple cancers and 77 6 years in those with quadruple cancers In the entire autopsy series 51 0 per cent were men 52 4 per cent of the subjects with single tumours and 65 6 per cent of those with multiple malignant tumours were men This preponderance of men was due to the high frequency of microscopically demonstrable prostatic cancer

In Table 2 are listed the number of various malignant tumours and their combination with multiple tumours The percentage figures are higher than those in Table 1 because tumours and not individuals are listed and besides in cases with double cancer both organs are listed in cases with triple cancer the three organs are listed and in cases with quadruple cancer the four organs are listed

Table 3 gives the locations (or types) and numbers of other cancers among the most frequent primary tumours (> 50 cases) Consequently the figures of total number are lower than those in Table 2

DISCUSSION

The occurrence of multiple malignant tumours in one and the same patient was first described in 1869 by *Billroth* and was published as a curiosity The cases published at the turn of the century were also described as rarities but in 1932 *Warren & Gates* whrote that Only that person whose experience with malignant disease is limited is now thrilled by encountering a case Multiple malignant tumours are thus not so rare as formerly believed and must be considered in clinical practice The majority of the many publications on this subjects are based on single cases on autopsy series clinical series or mixed series or on compilations of literature cases

The series by *Mersheimer et al* (1964) who collected data from the Connecticut Tumour Registry represent a community analysis They found 3 2 per cent incidence of multiple malignancy and their series probably represent one of the best available but they have many cases of multicentric cancer There are however recent studies (*e g Ferenczy* 1966 who described 1 004 cases diagnosed during 40 years) where nothing is said about the basic population or the autopsy frequency

The only reliable method to estimate the true frequency of multiple cancer is however within a defined population to perform thorough autopsies on subjects of whom detailed clinical records on previous diseases are available *Lombard et al* (1946) among others therefore omitted from their series patients who were alive because they found that the second tumour was detected at autopsy in half the cases (except in those with skin cancer) Too many authors have not payed due respect to this fact

The criteria of multiple cancers vary from author to author The most widely accepted criteria appear to be those used by *Warren & Gates* (1932) who state that the tumours should be histologically ma

lignant they should be separate and they should not be metastases In the present investigation we used an additional criterion namely that the tumours should be situated in different organs This was done because it is difficult or impossible to decide whether *more* than one tumour in a given organ should be regarded as multifocal primary tumour or metastasis As to primary liver cancer multifocal *origin* is probably common (*Elias* 1960) When the liver is *exposed to a carcino* genic agent it may be assumed that the entire organ is affected In the event of multiple foci in the liver it is however not possible to decide whether they should be regarded as multiple tumours or metastases This also applies to tumours of the urinary tract and of the colon and also to primarily generalized tumours such as the malignant lympho mas *Moertel* (1964)found the rate of occurrence of multiple primary malignant neoplasms to be 5 1 per cent but only 2 8 per cent if multi centric tumours of the same organs or tissues were excluded Only in 3 cases in our material were multiple tumours in paired organs not re garded as *one* tumour *vi* one case of bilateral mammary cancer where an adenocarcinoma appeared in the left breast 7 years after radical treatment of a carcinoma simplex on the right side (and without meta stases from any of the tumours at autopsy 3 years later) one case of bilateral bronchial carcinoma where one tumour was *an oat cell* carcin oma the other an adenocarcinoma one was a case of an adenocarcin *oma of the left renal pelvis and a carcinoma of transitional cell type* in the right ureter

Basal cell carcinomas were ignored firstly because of the benign nature of this lesion and secondly because it is *difficult to ascertain the frequency of previous basal cell carcinoma* in an autopsy series This difficulty also applies to squamous epithelial cancer of the skin In our series such tumours had doubtless occurred without being noted in the hospital records or so long ago that they were not covered by the files of the Department of Pathology It is also known that squamous epithe lial cancer is more common in clinical than in autopsy series (*Malmio* 1959)

Many of the tumours here designated as *metachronous have in fact* been synchronous since the second tumour(s) has been diagnosed clin ically or at post mortem shortly after radical treatment *of the first* tumour Since we know no safe method to decide whether a later diag nosed tumour was present at the time *when the first* tumour was cured the situation at autopsy was used

The frequency of multiple malignant *tumours* given in the literature varies widely (Table 4)

The frequency varies *between 0 5 to 11 7 per cent* (present material) This variation may be due to different factors *Different* authors use *different criteria for multiple cases* The series probably differ in age distribution Clinical series except those dealing only with skin cancers (*see Malmio* 1959) of course show a lower frequency and *in* the

autopsy series the type of hospital and the methods of autopsy (e g routine histologic examination of the prostate) and of selection of cases for autopsy will influence the results

TABLE 4

Frequency of Multiple Malignant Tumours in Autopsy and Clinical Materials

	Authors	Year	Total no investi gated	No of *malign* tumours	No of mul *tiple* tumours	Mult *malign* tumours in %
Autopsy series						
1	Feilchenfeld	1901	5 6??	507	10	2 0
2	Pedlich	1907	–	490	10	?0
3	Cade	1915	–	4 ?19	3?	0 8
4	*Harbitz*	1916	? 613	5?4	16	3 1
5	Rau	192?	10 393	1 13?	17	1 5
6	*Medi ed.en*	1924	4 638	470	?0	4 3
7	Junghanns	19?9	?6 049	4 ?19	19	0 5
8	Coriamowa & Schabad	19 0	6 65?	1 ?38	?3	1 9
9	Brandt & Jekabson	1830	14 893	2 083	11	0 5
10	Muller	1930	5 01?	1 171	19	1 7
11	Hanlon	1931	3 000	950	18	1 9
1?	Warren & Gates	193?	–	1 078	40	3 7
13	Burke	1936	2 033	583	46	7 8
14	Austin	19 8	8 124	887	23	2 7
15	*Kirshbaum* & Shively	1939	10 870	1 411	25	1 8
1?	Hellendall	1943	–	685	30	4 3
17	Warren & Ehrenreich	1944	–	2 829	194	6 8
18	Albrecht	1951	31 630	6 183	203	3 3
19	Fried	1958	4 800	1 514	24	1 6
20	Rae	19?8	? 130	711	60	8 4
?1	Moertel et al	1961	–	37 580	1 909	5 1
?2	Thoma	1964	2 346	–	–	4 3
?3	Present series	1968	11 100	4 895	572	11 7
Clinical series						
1	Hurt & Broders	1932	–	? 1?4	71	3 3
2	Schreiner & Wehr	1934	–	11 212	07	2 7
3	Desaive et al	1939	–	3 115	?6	1 2
4	Moder et al	19?2	–	3 996	179	4 5
5	Wallace	1957	–	3 006	1?4	4 5
6	Malmi	1959	–	27 71?	?30	2 3
?	Polk et al	1964	–	1 141	121	10 6

See *Malmio* (1959)

The highest frequencies of multiple cancer described in the literature are those reported by *Polk et al* (1964) 10 6 per cent basal cell carcinoma included *Rae* (19?8) 8 4 per cent microscopically demonstrable carcinoma of the prostate included and by *Burke* (1936) 7 8 per cent basal cell carcinoma included The high frequency reported by *Polk et al* (1964) is explained by the fact that they only studied the frequency of multiple cancers associated with carcinomas of the colon and of the rectum Since these carcinomas have a relatively good prognosis the

risk of developing a next tumour is greater than in many other tumours. To this must be added that basal cell carcinomas constituted more than one third of their multiple cases. They found that not less than one third of the patients who survived their first cancer for 5 years developed a new cancer. As mentioned our criteria for multiple cancer are more rigorous in that multiple foci in a single or in paired organs were regarded as one tumour. Even if latent prostatic cancer were not included in our series the frequency of multiple tumours would still be as high as 8.8 per cent.

The high frequency of malignancy 44.1 per cent among the autopsied subjects is a result of selection. In an attempt to ascertain the extent of the selection the records of all the 2 224 subjects who had died in 1965 in Malmo were analysed. Out of these 1 432 (64.4 per cent) were autopsied at the Department of Pathology and 752 malignant tumours were diagnosed in 645 subjects which is 45.0 per cent of those autopsied. Among the 792 not autopsied here 397 were autopsied at the Department of Forensic Medicine. Among these (many of them were young subjects) 33 malignant tumours were found. As regards the remaining subjects not autopsied death certificates were issued by general practitioners in private practice. By studying the death certificates and the files of the Department of Pathology it was found that 21 tumours had been diagnosed in these patients. An autopsy would of course have revealed a number of cancers not diagnosed *intra vitam*. Since the 54 tumours represent less than 10 per cent of those in autopsied subjects it would be better to correlate the autopsy material to the total number of deaths in the area. Since such studies are difficult and demand complicated statistical methods we intend to return to the problem in a later publication.

It is seen from Table 1 that approx. 10 per cent of all cancer cases had a second tumour, 10 per cent of those had a third tumour and 10 per cent of those had a fourth tumour. We are at the moment not able to explain this regularity (which may be incidental). There is no absolute correlation between the frequency of multiple cancer and mean age at death (Table 2). As a rule (if we exclude tumours constituting only a few cases) low mean age at death is associated with a low frequency of multiple tumours. This explains the low frequency of multiple tumours in cases of glioma. In cases of mesothelioma the frequency is very low despite the high mean age at death. This is probably due to the unwillingness of diagnosing a mesothelioma when the subject has another tumour which possibly could be responsible for the growth in the serous cavities. The high frequencies of multiple cancer associated with carcinomas of the prostate, kidneys and small intestines respectively depend on the large number of microscopically diagnosed prostatic tumours, that in the kidney cortical adenomas with a diameter of more than 2 cm were regarded as carcinoma and

that many small carcinoids of the small intestine were diagnosed incidentally at autopsy

In Table 3 the sites and types of primary cancers are given in the left column in order of decreasing frequency. When locations and types of the other tumour(s) are given in the same way it is seen that the distribution of multiple malignant tumours among the various organs of the body is approximately the same as that of solitary tumours

A question often discussed in the literature is whether the risk of development of a second tumour in a patient with one malignant tumour is greater than the risk of development of an initial malignant tumour in a given population *Peller* (1941) for instance believed that once a tumour had developed it would prevent immunologically the development of further tumours The frequency found in some series (*Muller* 1930 *Watson* 1953) and also in the present one lends no support to such an assumption Some authors (*Warren & Gates* 1932 *Bugher* 1934 *Warren & Ehrenreich* 1944) believe that the frequency of second and third tumours is higher than that ascribable to chance

Watson (1953) followed up 16 626 cancer patients for whom the man years exposure to the risk of developing a second cancer was computed and the author compared this risk with the risk which according to the figures for the normal U S population is involved He was unable to demonstrate any evidence of a constitutional tendency to develop a second tumour or of an immunity following the first

Malmio (1959) who includes multicentric cancer was not able to show that chances of contracting a new tumour were higher in patients with a single cancer than chances of such development in the population at large He found however like *Finhorn & Jacobsen* (1964) that multiple tumours were more common among young subjects than among elderly ones Our series does not seem to support this last finding Since multiple tumours in the same or in paired organs were excluded in our material (with the exception of three cases) it has not been possible to study whether the risk of development of a second tumour in an organ already harbouring a tumour is greater than the risk of development of an initial tumour in the same organ The existence of such a risk is however apparently well documented (*Lund* 1933 *Moertel & Soule* 1957 *Qualheim & Gall* 1957 *Moertel et al* 1961)

It is a well known fact that patients with multiple tumours survive longer than patients with only one tumour (*Warren & Gates* 1932 *Warren & Ehrenreich* 1944 *Lombard et al* 1946 *Malmio* 1959) but this seems only to means that the patients are apt to develop a new tumour because of their long survival

It would be interesting to know how often multiple tumours are of interrelated origin Theoretically one might imagine that certain tumours are hormone dependent and thereby apt to occur in association with one another *Moertel* (1964) was unable to demonstrate any such

association It is however accepted by *Fried* (1958) and *Hersheimer et al* (1961) who suggest that it may possibly be related to an endocrinologic factor

This problem is extremely difficult to analyse and we do not think that it is possible on the basis of our large series to give any definite answers It is seen from Table 3 that 22 other tumours were found among the 173 cases of ovarian malignancy Nine of these 22 i e 41 per cent were cancer of the breast while breast cancer is responsible for less than 20 per cent of all female cancer If we however look at the 478 cases of breast cancer it is seen that only 9 per cent of the other tumours are found in the ovaries which are responsible for 7 per cent of female malignancies This seems to indicate that subjects with ovarian cancer are apt to develop breast tumour but not *vice versa* The explanation might be that the serum estrogen level is high even in cases of non hormone producing ovarian tumours

It is important to remember as pointed out by *Clemmesen* (1965) that malignant diseases with a high cure rate when occurring early in life will offer a possibility for the development of a second cancer greater than that of other cancers To this comes the variation in risk with age for cancers at different sites

SUMMARY

In an investigation of the frequency of multiple tumours in a defined population (Malmo 250 000 inhabitants) where approx 60 per cent of all subjects are autopsied at death the following findings were obtained

1 Out of 11 098 patients examined *post mortem* 4 895 (44 1 per cent) had or had had 5 523 malignant tumours

2 Multiple tumours syn- or metachronous at autopsy were found in 572 subjects (11 7 per cent)

3 The types and locations of multiple malignant tumours were roughly the same as those of solitary tumours

4 The frequency of multiple tumours was with certain exceptions which are explained associated to mean age at death

5 The frequency of multiple tumours increased with duration of survival after treatment

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THE TWO TYPES OF A CELLS IN THE ISLETS OF LANGERHANS OF NORMAL AND SYNTHALIN TREATED GUINEA PIGS

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The morphological and functional characterization of the various cell types of the islets of Langerhans has been a matter of growing interest during recent years. It has been shown that the islets of most species studied up to now contain in addition to the insulin producing B cells also A_1 cells displaying an argyrophil reaction with a modified Davenport technique and A_2 cells which lack this property (*Hellerström & Hellman* 1960, *Hellman & Hellerström* 1969). Some authors claim that the A_1 cell and the islet D cell of *Bloom* (1931) and *Thomas* (1937) are identical (*Epple* 1964, *Solcia & Sampietro* 1965, *Fujita* 1964, 1968, *Tillbach* 1968), whereas others do not accept this view without reservation (*Hellerström & Asplund* 1966, *Björkman et al.* 1966, *Östberg et al.* 1966). This controversy seems now to depend mainly on the observation that in the islets of foetuses and certain lower vertebrates the classical granule stains fail to differentiate between the granular D cells and the agranular islet cells (*Björkman et al.* 1966, *Östberg et al.* 1966, *Epple* 1968).

While it seems well established that glucagon is elaborated by the A_2 cells (*Unger et al.* 1967), the biological significance of the A_1 cells is still an enigma. It is not even known with certainty whether the latter cell type should be regarded as functionally independent or indeed if it exhibits any secretory activity at all. Evidence against the view that the A_1 cell is a particular functional stage of another islet cell type has been derived from its specific histological and histochemical staining characteristics and its reactions to various experimental procedures (cf. *Hellerström et al.* 1964, *Hellman & Hellerström* 1969). On the other hand mixed forms of A_1 cells and A_2 cells have been reported in some studies (*Björkman et al.* 1966, *Björkman & Hellman*

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1964 *Erbengi* 1964) The present investigation was carried out to evaluate further the specific properties of the two types of A cells by analysing these cells after administration of an A cytotoxic agent Synthalin A to guinea pigs

MATERIAL AND METHODS

Altogether 75 male guinea pigs weighing 200–700 g were used The animals had free access to water and a diet consisting of hay root vegetables and pellets Synthalin A (decamethylenediguanidine dihydrochloride L. Light & Co Ltd Colnbrook England) was dissolved in sterile isotonic saline to a concentration of 1 mg/ml and injected subcutaneously The solution was given either as a single injection (3 3 5 mg/kg b w) or as one daily injection (2 mg/kg b w) for three subsequent days (Table 1) The animals were killed by a blow to the head and exsanguinated 44–77 hours after the first injection Control animals were treated similarly except that injections consisted of sterile isotonic saline only

At various intervals during the experimental period blood samples were obtained from the ears and at the time of killing from the severed neck vessels The blood glucose concentration was determined with the glucose oxidase method as described by *Hjelm & de Verdier* (1963) After death the pancreas was rapidly removed and small pieces of tissue were either frozen at −70 C in isopentane or fixed at room temperature in Zenker formol solution (*La arus & Volk* 19??) The fixed tissue specimens were dehydrated and embedded in paraffin or Epon 812 (*Luft* 1961) Cryostat microtome sections 10 μ thick were obtained from the frozen blocks and immediately studied in dark field illumination Sections 4 or 7 μ thick were cut from the paraffin embedded material and either silver impregnated (*Hellerström & Hellman* 1960) or granule stained with chrome haematoxylin (*Bencosme* 195?) or aldehyde fuchsin In both these latter cases the counter stain was ponceau fuchsin In addition some sections were stained with phosphotungstic acid haematoxylin (PTAH *Lillie* 1965) The Epon-embedded tissue was cut into sections 0 5–1 μ thick which were silver impregnated or stained with aldehyde fuchsin ponceau fuchsin after removal of the plastic with an alcoholic NaOH solution (*Ian & Europa* 1965) Classification of the two types of A cells was accomplished by photographs of silver impregnated islets followed by removal of the silver and re staining with one of the granule stains (*Hellerstrom & Hellman* 1960)

TABLE 1

Dosage of Synthalin A and Survival Rate in the Different Groups of Experimental Animals

Number of animals	Synthalin A (mg per kg b w at each injection)	Number of injections	Number of animals surviving after 3 days
14	2 0	3	14 (100 %)
22	3 0	1	13 (59 %)
14	3 5	1	6 (43 %)

The content of refractile silver white granules in the cytoplasm of the A_2-cells was graded semiquantitatively in dark field illumination of the cryostat microtome sections the microscopist being confronted with "unknown" sections The procedure was applied to pancreatic material from 7 control animals and 9 animals which had received daily injections of Synthalin A (2 mg/kg b w) during 3 consecutive days All of these animals had been killed 24 hours after the last injection

Quantitative histological analyses were performed in 8 Synthalin treated guinea pigs and 9 control The treated animals had received the drug in different dosages according to Table 1 and were selected among those animals which had clear mor



Figs 4–5

Fig 4 Detail of a vacuolated A cell in a thin chrome haematoxylin ponceau fuchsin stained section from Fl n embedded pancreatic material Some (acidophil) granules may be distinguished in the remaining cytoplasm (arrows) × 2000

Fig 5 Mitotic figure in an islet cell which may represent a vacuolated A cell No distinct (acidophil) cytoplasmic granules could be identified Technique as in Fig 4 × 2000

Synthalin injected animals contained obviously enlarged nuclei with decreased density of the chromatin net Pycnotic nuclei were seldom seen in the injured cells In addition mitotic figures were recorded in a few islet cells which however could not be classified according to type (Fig 5)

TABLE 2

Effects of Synthalin A Treatment on the Percentage Contribution of A_1 Cells to the Total Islet Volume and on the Mean Nuclear Size of the Different Islet Cell Types Means ± S E M

Animal group	A_1 cells %	Nuclear size (Arbitrary units) A_1 cells	A cell	B cells
Controls (9)	10.4 ± 0.9	1.1[illegible] ± 0.05	1.[illegible]9 ± 0.03	1.17 ± 0.04
Synthalin A treated (8)	8.8 ± 0.[illegible]	1.09 ± 0.05	1.65 ± 0.10	1.11 ± 0.05

The quantitative histological methods were applied to those Synthalin treated animals in which there were A_1 cells displaying clear morphological changes As can be seen in Table 2 the percentage contribution of A_1 cells to the islet volume in the treated animals did not differ

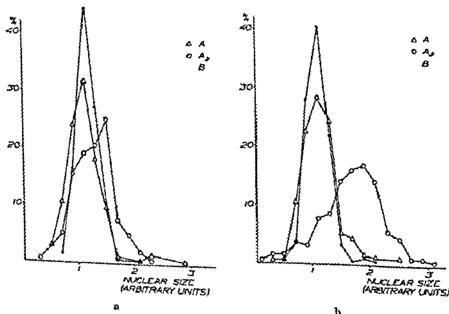


Fig 6

a) Size frequency distribution of the nuclear section areas from the various islet cell types in normal guinea pigs b) A corresponding plot from guinea pigs treated with Synthalin A It is evident that in these animals the peak of the A -cell curve is displaced to the right and that the curve is considerably broadened

from that in the control animals ($t = 1\ 32$ $P > 0\ 05$) Likewise there was no difference between the two groups in the nuclear size of the A_1 cells ($t = 0\ 44$ $P > 0\ 05$) or the B cells ($t = 1\ 26$ $P > 0\ 05$) By contrast the nuclear size of the A cells was significantly increased in the Synthalin injected animals ($t = 3\ 71$ $P < 0\ 01$) When the nuclei of the various islet cell types were classified according to size the frequency curves on the whole showed symmetrical distributions (Fig 6) In the Synthalin treated animals the peak of the A cell curve was displaced towards higher values and the base of the curve was considerably broadened

DISCUSSION

It emerged from the present study that all those cells which exhibited cytoplasmic vacuoles and degranulation after administration of Synthalin A should be classified as A cells In addition quantitative analyses showed that changes in nuclear size were confined to this cell type A lack of effect on the A cells was further indicated by the finding that these cells contributed equally to the islet volume in the two animal groups These observations confirm and extend previous reports indicating a selective effect of Synthalin A on the pancreatic islet A-cells (*Davis* 1952 *Korp & Le Compte* 1955 *Creutzfeldt & Tecklenborg* 1955 *Munger* 1962 *Solcia & Sampietro* 1965) Although the present

data support the view that the A_1 and A_2 cells are physiologically separate entities it cannot as yet be excluded that these cell types represent different stages in a common functional cycle It has been reported for example that islet B cells with a relatively low rate of insulin secretion are much more susceptible to the toxic action of alloxan than those with a high functional activity (*Lazarus & Volk* 1962)

Little is known regarding the mode of action of Synthalin A on the islet A_2 cells It has been suggested that administration of the drug brings about an increased functional demand on these cells some of which may be exhausted as evidenced by the presence of cytoplasmic vacuoles (*Creutzfeldt* 1960) A more direct cytotoxic effect of Synthalin A has also been proposed and the hypoglycaemic effect of the drug was then assumed to reflect a deficiency of glucagon (*Holt et al* 1955) In the present study the A_2 cells displayed both degranulation and an increased mean nuclear size whereas pycnotic cell nuclei were rare even in severely vacuolated cells These observations seem to corroborate the view proposed by *Hultquist* (1959) and *Creutzfeldt* (1960) that the A_2 cells respond to Synthalin A with increased functional activity and sometimes exhaustion rather than cytotoxic degeneration

In addition to Synthalin A and some other guanidine derivatives (*Creutzfeldt & Moench* 1958) certain salts of heavy metals particularly cobaltous chloride have been found to be injurious to some A type cells of guinea pig islets (*van Campenhout & Cornelis* 1951) The lesions brought about by these agents appear morphologically very similar to those observed after administration of Synthalin A Moreover the effect of cobaltous chloride also seems to be confined to the glucagon producing A_2 cells (*Petersson et al* 1962) This is further supported by the recent observation of a marked increase of the plasma glucagon activity after administration of this compound to rats (*Lochner et al* 1964) Whether there is a common mechanism for the effect of guanidine derivatives and certain metal salts on the A_2 cells remains a matter of speculation It is worthy of note that in the guinea pig cobaltous chloride does not seem to decrease the blood sugar level (*Creutzfeldt & Schmidt* 1954) whereas Synthalin A brings about a characteristic hypoglycaemic state

SUMMARY

Light microscopical analyses of the two types of pancreatic A cells were performed after subcutaneous administration of Synthalin A to guinea pigs In about 50 per cent of the treated animals a moderate number of islet cells showed a conspicuous cytoplasmic vacuolization By analysing pancreatic sections which had first been silver impregnated and later after removal of the silver restained with one of the granule stains it was found that the affected cells should be classified as A_2 cells no morphological changes in the A_1 or B cells were recorded In addition the A_2 cells showed an increased mean nuclear size

and a marked degranulation after Synthalin A The results further support the view that the A_1 and A_2-cells are physiologically separate cell types

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FINE STRUCTURE OF ARTERIOSCLEROSIS INDUCED IN RABBIT AORTA BY EPINEPHRINE AND THYROXINE

By

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Received 5 xii 68

Intravenous injection of epinephrine in the rabbit produces arteriosclerotic lesions which are considerably aggravated by simultaneous administration of L thyroxine An increase in the mucopolysaccharide content and in the uptake of ³S with unchanged hydroxyproline content was found in such conditions by *Lorenzen* (1959 1961) The technique of electron microscopy of mucopolysaccharide molecules is insufficiently developed The present electron microscopical study was intended to reveal epinephrine induced lesions in the rabbit aorta The experimental procedure was similar to that used by *Lorenzen* (1959 1961)

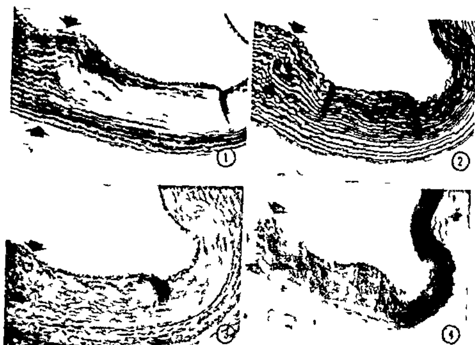
MATERIAL AND METHODS

Through one week three white male rabbits (body weight approximately 2 kg) had daily intravenous injections of epinephrine During the first five days the dose was increased gradually from 0 075 mg/kg to 0 050 mg/kg L-thyroxine 0 05 mg/kg daily was injected subcutaneously in an aqueous suspension through the same period (*Lorenzen* 1961) After one week the rabbits were killed by intravenous injection of 200 mg of Nembutal® The thoracic aorta was removed. The intimal surface exhibited three kinds of gross changes i e grossly normal nodular and paper like areas Specimens were removed from these areas and fixed in an ice-cold 1 per cent osmic acid solution of veronal acetate buffer pH 7 2 After dehydration in graded alcohols specimens were stained with a 1 per cent phosphotungstic acid solution in absolute alcohol and embedded in prepolymerized methacrylate Ultra thin sections (about 500 Å) were cut by an LKB ultramicrotome and observed by a Philips 100 B electron microscope at 60 kV For histological study 3 μ thick sections were cut of the same specimens by the same ultramicrotome After removal of the methacrylate by acetone the thick sections were stained by haematoxylin eosin resorcin fuchsin toluidine blue and calcium red (*McGee Russell* 1955)

RESULTS

Gross findings The inner surface of the thoracic aorta of all animals exhibited nodules and plaques The nodules were of about five milli

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Figs 1 2 3 and 4

Specimens from a paper like area Arrows indicate the borderlines between the plaques (right) and the grossly normal areas (left) 1 Toluidine blue staining 2 Acid orcein staining 3 Haematoxylin eosin staining 4 Calcium red staining Fig 1 shows unstained elastic lamellae The medium dark areas between the elastic lamellae are the metachromatically stained extracellular spaces In Fig 2 the elastic lamellae of the diseased area show no wavy appearance Fig 3 shows very few nuclei in the media of the diseased area and numerous spindle shaped nuclei outside the lesion Fig 4 Calcified areas are stained by calcium red

metres in diameter oval cloudy slightly elevated and well demarcated against the surrounding smooth surfaces The plaques were irregularly oval with a long axis of one or two centimetres not elevated and with a paper like wrinkled surface

Histological findings After toluidine blue staining all smooth muscle cells of the grossly normal areas of the aortic wall appeared blue stained The spaces between these smooth muscle cells and the elastic lamellae were stained metachromatically (Fig 1) No changes were found in the endothelial cells nor in the elastic lamellae In nodular areas with a smooth surface and areas with a paper like wrinkled surface the smooth muscle cells appeared like those of the grossly normal areas However the elastic lamellae were arranged straight and parallel and each of them revealed thinning s and fragmentations (Fig 2)

The endothelial cells were flat and showed desquamation in certain areas These changes were more severe in the paper like wrinkled areas than in the nodular areas Very few nuclei were found in certain lesions which were surrounded by numerous spindle shaped nuclei (Fig 3) In



Fig 5

A grossly normal area of aortic wall Two smooth muscle cells in the centre of the photograph are oval and contain nucleus (N) mitochondria (M) and vacuoles (V) Arrows indicate the connections of these cells to elastic fibres In the right side of the photograph a smooth muscle cell shows an exceptionally dense cytoplasm Dense thread like material is seen on the surfaces of the elastic lamellae (EL) In the space between the lamella and the smooth muscle cells a dense granular material (G) and collagen like fibrils (C) can be seen × 8 400

the paper like area calcium deposits were demonstrated in the media by calcium red staining (Fig 4)

Electron microscopical findings In the grossly normal areas some smooth muscle cells of the inner and middle layers of the media were oval with vacuoles (Fig 5) or cystic cytoplasmic protrusions containing fine granular or thread like material (Fig 6) Others showed a dense cytoplasm consisting of myofilaments vacuoles and mitochondria An attachment of smooth muscle cells to elastic lamellae was seen in a few areas The elastic lamellae showed no internal changes but the surfaces of the lamellae and their branches showed dense fine granular material or thread like figures (Fig 7) The spaces between the smooth muscle cells and the elastic lamellae were filled with dense fine granular material dense elastic filaments and collagen like fibres the latter



Fig 6

A grossly normal area of aortic wall Note large cystic protrusions of the sarco plasm of a smooth muscle cell (CP) Arrow indicates the connection between these protrusions and degenerated elastic fibres The elastic lamella (EL) and the elastic branch (E) show dense fine granular material on their surfaces (G) This material is also seen in the spaces between the elastic fibres and the cystic protrusions of the smooth muscle cells There are collagen like fibril (C) in the same space
× 14 800

showing no transverse banding (Fig 7) The outer parts of the media showed changes similar to those of the inner and middle layers The endothelial cells contained nuclei mitochondria and vacuoles and showed clear intercellular connections However the elastic fibres anchoring the endothelial cells appeared granular like the fibres between the lamellae (Fig 8)

In the nodular and paper like areas the endothelial cells were flat or oval containing a nucleus while other cell organelles were indistinguishable (Fig 9) The connections between two neighbouring endothelial cells were distinct and the anchoring elastic fibres were granular similar to those of the apparently normal area (Fig 9) The smooth muscle cells below the endothelial cells contained dense granu



Fig 7

A grossly normal area of aortic wall Between the elastic lamellae (EL) a smooth muscle cell containing vacuoles mitochondria and myofilaments is shown It has direct connections to dense elasti fibres (arrows) Dense fine granular material is found on the surface of the elastic lamellae (framed arrows) In the spaces between the elastic lamellae and the smooth muscle cell note numerous collagen fibres (C) dense elastic fibres (DE) and dense granular material (G) × 8 5?0

lar material but myofilaments or other cell organelles could not be identified Between the elastic lamellae of the media the smooth muscle cells showed compact masses of myofilaments The elastic lamellae were straight and parallel (Fig 9) The spaces between the elastic lamellae and smooth muscle cells were filled with collagen like fibrils and degenerated elastic fibrils The outer area of the media contained spindle shaped fibroblasts (Fig 10) In the paper like area calcium precipitates were found as extremely dense granular masses and needle like crystals on the surfaces of the elastic fibres (Fig 11)

DISCUSSION

The main alterations in rabbit arteriosclerosis induced by epinephrine plus thyroxine occurred in the smooth muscle cells and the elastic fib



Fig 8
An endothelial cell of a grossly normal area Note nucleus (N) mitochondria (MI) and small vacuoles (V) The anchorings under the cell appear destroyed and dense elastic fibres (DE) are seen Note connections to the adjacent endothelial cells (arrows) × 14 600

res of the aorta very much like the lesions seen in arteriosclerosis of man (*Geer et al* 1961 *Kawase* 1963 *Daoud et al* 1964 *Haust et al* 1967) and domestic animals (*Kawase* 1963 *Still* 1964 *Dahme* 1965 *Geer* 1965 *Parker et al* 1966 *Kniericm* 1967) In the present study there were no foam cells with fat droplets like those seen in human atherosclerosis but the smooth muscle cells revealed structural changes such as cystic protrusions of the sarcoplasm vacuole formation and condensation of the myofilaments

As to the granular or thread like material of the extracellular spaces *Haust et al* (1965) using material from human atherosclerosis and electron microscopic immunohistochemical technique demonstrated the presence of fibrin *Haust et al* (1967) believed that the lipid components of human atherosclerosis coexisted with degenerated elastic fibres remnants of fibrin or detached basement membrane of smooth muscle cells In the present study a minor part of the dense thread like



Fig 9

Paper like aortic area The endothelial cells (E) are flat showing no clear cell organelles or anchorings There are connections with the neighbouring endothelial cells (arrows) The smooth muscle cells (M) reveal dense masses of myofilaments and a complete loss of connections with elastic lamellae The elastic lamellae (EL) are straight Dense elastic fibres (DE) and collagen like fibres (C) are located in the extracellular compartments × 8 500

material along the elastic fibres and in the area of degenerated smooth muscle cells may represent degenerated elastic material and myofilaments However most of the granular and dense thread like material could not be identified as such structures nor as remnants of fibrin or all mixed together The numerous collagen like fibrils in the extra cellular spaces showing no distinct transverse bands were parallelled by increased total contents of hydroxyproline in the biochemical studies of *Loren en* (1959 1961)

Degeneration of elastic fibres may give rise to disanchoring of the endothelial cells and smooth muscle cells (*Bierring et al* 1963) It may cause desquamation of the endothelial cell layer and together with changes of smooth muscle cells loss of waviness of elastic lamellae The areas where the dense thread like and granular material were found

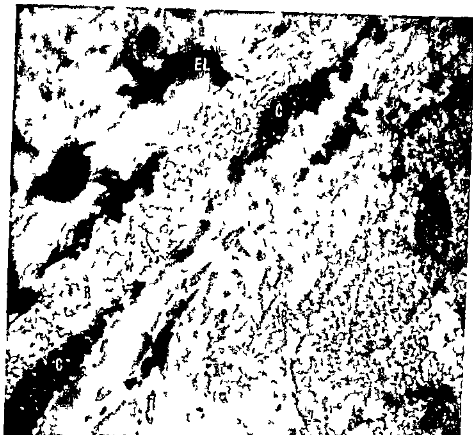


Fig 10

Outer part of the media in a paper like area Cells containing endoplasmic reticulum (R) probably fibroblasts are seen between the elastic lamellae (El) In the extracellular space there are collagen like fibrin (C) × 27 600

were the same as those staining metachromatically However the dense material could not be identified with certainty as the acid mucopolysaccharides demonstrated earlier by histochemical and biochemical methods

The same type of calcification demonstrated on the surfaces of the elastic fibres has previously been found after three weeks in epinephrine influenced rabbit aorta by Lu *et al* (1965)

In atherosclerosis of man and domestic animals the pathogenetic role of the endothelial cells has been discussed by *Dahme* (1965) *Geer et al* (1961) *Geer* (1965) and *Still et al* (1964) In the present study on arteriosclerosis the endothelial cell changes seemed to be less significant than those in atherosclerosis



Fig 11

Calcification of elastic lamellae (EL) Extremely dense granular precipitates (GR) and needle like crystals (CR) are seen This area showed intense red histochemical stainability with calcium red × 85 800

SUMMARY

Experimental arteriosclerosis induced in the rabbit by epinephrine plus thyroxine was studied by electron microscopy and ordinary light microscopy The manifest changes were degeneration of elastic fibres cystic protrusions from and vacuoles in the sarcoplasm and condensation of myofilaments of the smooth muscle cells Calcification of the elastic fibres and degeneration of the endothelial cells were also found

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Acta path microbiol scand. 76 203-214 1969

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THE PULMONARY VASCULAR PATTERN IN IDIOPATHIC RESPIRATORY DISTRESS

A Micro Angiographic Study

By

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Received 13 xii 68

The Idiopathic Respiratory Distress Syndrome (IRDS) is associated with the formation of air space lining membranes made up of blood protein (5) This circumstance together with the recent observation that pulmonary ischaemia is a component in the clinical entity (1 2) has inspired a steadily increasing interest in the structural appearance of the microcirculation of the lungs in IRDS

Micro angiographic studies of the pulmonary circulation in the perinatal period illustrate the pulmonary vascular bed down to the capillary level (18) and is a suitable tool for this purpose in IRDS studies (9 12) It has been suggested (9) that the precapillary and capillary filling is reduced in pulmonary microradiograms from IRDS infants

The post mortem lung of infants dying from IRDS is almost invariably collapsed due to terminal resuscitation efforts with oxygen In view of the fact that structural changes are known to occur in the vascular tree when the lung expands (17) and the vascular resistance is known to diminish when the collapsed lung is aerated (4) it would seem desirable to investigate lungs from infants with IRDS with a technique that permits the injection of an isotonic contrast medium into the pulmonary circulation when the organ has been adequately aerated or preferably is rhythmically ventilated during the injection This would serve the double purpose of making the IRDS lung more "normalized" with respect to the architecture of the vessel as well as promote the filling of the smaller vessels

The present investigation was undertaken in order to evaluate possi

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This investigation has been supported by grants from The Swedish National Association against Heart and Chest Diseases Stiftelsen Therese och Johan Anderssons Minne and Stiftelsen Gustaf och Tyra Svenssons Minne

MATERIAL

Autopsy specimens from 19 perinatal cases were used for the experiments *Five pairs of lungs were obtained from non macerated stillborn* infants (birth weight *1 910–4 060 grams) six pairs of lungs from newborn* infants who died from extra pulmonary causes and who had no evidence of cardiovascular malformations (*birth weight 1 140–3 150 grams* survival time 10 minutes–4 days) *eight* pairs of lungs *were derived from infants with respiratory distress* in all of whom hyaline membranes were histological *demonstrable (birth weight 1 150 ? 890 grams* survival time 10–48 hours) *In all groups about half of the* specimens had been kept deep frozen at —?0 during preparation for this study The *specimens were thawed at room temperature At* autopsy great care was taken not to cut into the lung parenchyma and the specimens used for the study were all intact In the last two groups the material was obtained less than 20 hours after death

RESULTS

Micro Angiography

When micro angiograms were studied without knowledge of the clinical data it was not possible to delineate specific vascular patterns for any one of the groups The only constant finding within all groups was that the filling increased with maturity as reflected in the birth weights

All specimens were found to be technically adequate The pulmonary artery and its branches including the arterioles were usually filled In some regions in all specimens there was also capillary filling In none of the specimens injected from the arterial side was there any filling of the pulmonary or bronchial veins *Aeration of the lung caused* a structural change of the vascular tree owing to the intermixing of air filled spaces but respiratory *movements during* contrast injection did not seem to enhance capillary filling nor was the presence of arterio venous shunts revealed

In view of findings by other workers in this field (9) a different pattern was expected in the various groups of cases Specifically we expected to find a poor outline of the pulmonary vasculature in the case of respiratory distress In fact this was not the case In the early phases of the experiment occasional instances of poor filling of the pulmonary arterioles were found in lungs from cases of respiratory distress but later similar instances were encountered in cases of still

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Figs 2–3

Fig 2 *Hyaline membrane disease* Micro angiograms (PA injection) from two specimens *without ventilation* A Birth weight 2 ?00 g gestational age *36 weeks* death at ?4 hours *Poor fillin of* arterioles and capillaries *in left lower lobe* × 1? B Birth weight ? 170 g gestational age 34 week death at 24 hours Good filling of arterioles and capillaries of right lower lobe × 1?

Fig 3 *Hyaline membrane disease* Micro angiograms (PA injection) from two specimens *after ventilation* 30 minutes negative pressure (*see text*) A Same case as illustrated in Fig ? A Left upper lobe was ventilated and shows the same appearance as the non ventilated left lower lobe (Fig ? A) *with poor* filling × 12 B Same case as illustrated in Fi 2 *B Moderate filling of left* lower lobe ventilation × 1?


2a
2b
3a
3b


4b
5a
5b

Fig 6

Micro angiography after injection into only pulmonary veins A Newborn birth weight 2 730 g gestational age 37 weeks death at 4 days Atresia of aortic arch Normal pattern × 12 B Hyaline membrane disease Birth weight 1 690 g gestational age 31 weeks death at 5 days Normal pattern including peribronchial venous plexa × 12



Figs 4–5

Fig 4 *Stillborn infants* Micro angiograms PA injection after ventilation (A) and without preceding ventilation (B) A Non macerated twin A birth weight 2 200 g donor in placental transfusion syndrome Good filling of arterioles and capillaries after negative pressure breathing × 12 B Non macerated stillborn, birth weight 4 000 g tentorial tear Poor filling of capillaries and arterioles Injected without preceding ventilation × 12

Fig 5 *Newborn infants without pulmonary disease* Micro angiograms (PA injection) after ventilation (A) and without ventilation (B) A *Birth weight* 3 100 g gestational age 38 weeks age at death 24 hours Traumatic bilateral adrenal haemorrhage Right upper lobe was ventilated before micro angiography Poor filling of arterioles and capillaries × 12 B Same case as in A Non ventilated right lower lobe shows good filling × 12

birth and newborns without evidence of hyaline membrane disease The vascular pattern in the three groups is illustrated in Figs 2–6

With the technique used no arterio venous anastomoses were found The same number and type of anastomoses between the bronchial and pulmonary arteries as described by *Robertson* (18) were encountered in all three groups In a few cases in each group pulmonary venous injection was performed The venous micro angiograms produced did not show any difference between the groups (Fig 6)

Histology

The microscopic sections from the micro angiographed blocks confirmed that no ruptures had occurred during injection In four cases there was interstitial emphysema In serial sections no arterio venous communications could be demonstrated All specimens from cases of clinical respiratory distress showed the presence of hyaline membranes In no instance was there any inflammatory reaction The *lymphatics* of the lungs were studied systematically in all sections in view of the interest in the lymph flow of the lung in respiratory distress which recently has emerged (11 16 25) The perivenous lymphatics were markedly widened in all lungs with *hyaline membranes* This was also the case in the lobes of such lungs as had not been ventilated post mortem The lungs from newborn infants *without pulmonary disease* had negligible dilatation of the pulmonary lymphatics In one of these cases there was a difference between ventilated and non ventilated parenchyma inasmuch as the non aerated lobes showed moderate lymphatic dilatation while the aerated lobes did not show such dilatation a fact that might mean that ventilation may assist in drainage of pulmonary lymph The series of lungs from *stillborns* showed no dilatation of lymphatics regardless of whether or not the lobes had been ventilated post mortem

COMMENT

The histological examination of the lungs following micro angiography confirmed that the respiratory distress group contained only cases with non inflammatory hyaline membrane disease without aspiration the live newborns without IRDS showed no pulmonary changes except final interstitial haemorrhage and the stillborns had no appreciable pulmonary changes such as aspiration etc The four cases with experimentally induced emphysema belonged in the group of stillborn (3 cases) and newborn infants without IRDS

Although the series is small the vascular pattern of the lungs from the three groups seem worthy of a comparison The microscopical sections had the well known characteristics of pulmonary structure in respiratory distress The dilatation of pulmonary lymphatics in IRDS-lungs in man as described by *Wade Evans* (25) and *Lauweryns et al*

(10 11) and in lambs as described by *Normand et al* (16) was con firmed This lymph angiectasis was prominent in all such lungs and it was minimal or absent in the two other groups *with one exception* This was the lung from a live born without IRDS which had moderate dilatation of lymphatics in a lobe that had not been ventilated during the experiment but which had normal lymphatics of the aerated lobes The possibility of artifact seems to be remote since a) Rheomacrodex was used in the contrast medium instead of water and b) the remain ing 9 specimens without hyaline membranes did not show lymphan giecta is It seems possible that decreased ventilation interferes with the drainage of lymph as suggested by *Schulz* (20) in a discussion of the electron microscopic findings of lymphangiectasis in lungs after extracorporeal circulation That a similar explanation should pertain to the constant finding of lymphangiectasis in the IRDS lungs seem less likely The cause of the—apparently experimentally inversible— lymphangiectasis in the IRDS lungs still remains to be explained

Likewise the arterial pattern in lungs with hyaline membrane dis ease has been interpreted differently by several workers Some con sider a premature dilatation of the pulmonary vessels the cause of respiratory distress (24) others consider the pulmonary arterial media to have normal thickness *i e* neither dilated nor constricted (8) and still others describe marked constriction (15 22) and in lambs even arteritis of the small arterioles (22)

However the vascular pattern as revealed by our micro angiography studies showed no appreciable difference between the groups At an initial stage of our experiments immature IRDS lungs showed areas with poor arteriolar and capillary filling This observation was con veyed to *Dr Lauweryns* (11) However as our study progressed it became evident that areas of such incomplete filling occurred in lungs from all groups particularly in immature lungs In essence then our conclusion is that there is no anatomical difference in the post mortem pulmonary vascular pattern in IRDS as compared with normal lungs of comparable gestational ages This statement includes arterial ana stomoses which were of the same number and appearance as in normal lungs described by *Wagenvoort & Wagenvoort* (26) and *Robertson* (18)

Whether the differences in technique used in *Lauweryns* and our experiments can explain the discrepancy in the results is difficult to evaluate *Lauweryns* used a thick water suspension of barium sulphate (40 g/60 ml) compared to ours (7 5 per cent barium sulphate) in 6 per cent Rheomacrodex Our medium had a pH of 4 8 a value that *should favour* "vasoconstriction rather than dilatation The pulsatile flow that we used is perhaps comparable to his continuous flow in 2 min increments but might explain our better filling An additional and essential difference in experimental model is that our controls con

sisted of human lungs identically prepared while *Lauweryns* used lungs of lambs

Our main findings of anatomically normal arterial pulmonary vascular pattern in IRDS of course reflects the conditions post mortem It has no bearing on the previous existence of an *in vivo* vasoconstriction as the background of a hypoperfusion in IRDS (1, 2 14) Since the pulmonary circulation is under nervous and humoral control (3) and true pulmonary ischaemia in IRDS in all probability is mainly secondary to functional constriction it would seem natural that post mortem injection studies would fail to reveal the structural background of an underperfusion To study such a mechanism *in vivo* experiments seem desirable Recent attempts at micro angiography on living normal piglets have been successful (7)

The controversy concerning the structural background of the large right to left shunt known to be present in IRDS (19 23 27) should be settled for the time being The absence of arterio venous or large artery artery anastomoses in the human lung in IRDS as shown by micro angiography is noteworthy and the only explanation at present of a R L shunt *in the lung* is a passage of blood through non or poorly ventilated parenchyma The observations of arterio venous communications in the lungs by *Gromowski* (6) remain to be confirmed *Sobin et al* (21) using a silicone elastomer microvascular casting method did not find such anastomoses

It has been demonstrated here that there is no micro angiographical evidence of differences in pulmonary vasculature after pulmonary artery or pulmonary vein injection of lungs from infants with IRDS newborn infants without pulmonary lesions or stillborn infants This method of study is a valuable tool in many vascular contexts but for the vital question of the pathogenesis of IRDS functional and biochemical rather than morphological approaches to studies of the pulmonary vasculature and alveoli now appear to be more promising

SUMMARY

Nineteen pairs of lungs from perinatal autopsy cases were used for a standardized micro angiographic examination A contrast medium of 7 5 per cent barium sulphate in 6 per cent Rheomacrodex was injected with pulsatile flow for 30 minutes while the lungs were ventilated with negative pressure in a chamber After formalin fixation all lobes were micro angiographed and serially sectioned for microscopic examination The case series consisted of 5 pairs of lungs from stillborns 6 from newborns born alive and without pulmonary disease and 8 from infants dying from respiratory distress syndrome (IRDS) and presenting hyaline membranes The pulmonary arterial pattern was essentially similar in all groups No arteriovenous anastomoses were seen The filling of precapillary arterioles and capillaries was equally good in

ventilated and collapsed lung specimens There seems to be no vascular anatomical prerequisite in the neonatal lung for the development of IRDS

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EFFECTS ON THE ENDOCRINE PANCREAS IN CHINESE HAMSTERS FED ZINC DEFICIENT DIETS

By

LENNART BOQUIST and ÅKE LERNMARK

Received 19 xii 68

Zinc occurs in the endocrine pancreas of many species (*Maske* 1957 *Voigt* 1959) Following the discovery by *Scott* (1934) of the role of zinc for the crystallization of insulin the physiological relationship between zinc and insulin has been the subject of several investigations (*cf* *Vallee* 1959) It has been assumed that zinc is concerned with the occurrence or storage (*Maske* 1953 1957 *Logothetopoulos et al* 1964) and secretion (*Maske* 1953 1957) of insulin in the β cells The role of zinc in these processes remains however poorly understood (*Vallee* 1959 *Grodsky & Forsham* 1966) An association between zinc and glucagon in the islets has also been suggested (*cf* *Vallee* 1959) In diabetic patients an increased urinary excretion of zinc has been observed (*Tarui* 1963b) but it has not been possible to decide whether such hyperzincuria is the cause the consequence or an insignificant concomitant symptom of diabetes mellitus (*Constam et al* 1964) Increased concentration of zinc in the urine has also been found in alloxan diabetes (*Tarui* 1963a) The plasma zinc content in diabetics without complications has been reported to be increased (*Constam et al* 1964) or unchanged (*Wikbladh* 1951 *Prout et al* 1960 *Craig* 1962 *Davies et al* 1968) as compared with normal subjects *Todd et al* (1934) showed that zinc was essential in diets fed to rats Deficiency of zinc in this species has been observed to give impaired glucose tolerance (*Hove et al* 1937 *Quarterman et al* 1966) To the best of our knowledge there are no previous studies on the intravenous glucose tolerance of zinc deficient animals

The main purpose of the present work on the Chinese hamster was

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21 [illegible] "[illegible] pattern" [illegible] pulmonar[illegible] Dis [illegible] 235 19[illegible]

5 [illegible] The formation of hyaline [illegible] Arch Dis [illegible] 2

6 [illegible] Arteri[illegible] arteries and pulmobronchial arteries 13 [illegible] 19[illegible]

2 Wallgren [illegible] Hansson [illegible] studies of the human [illegible] 196[illegible]

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concentration before and rather soon after the administration of glucose the blood was sampled at 0 and 30 minutes of the experiments The principles of the blood glucose determinations were the same as those previously described (*Boquist* 1967 c) For insulin assay the blood was collected in polyethylene micro tubes and allowed to coagulate at +8 C before centrifugation Then the serum samples were frozen and stored at —25 C The serum insulin level was determined with the double antibody radioimmunological technique (method C) of *Hales* & *Randle* (1963) Crystalline ox insulin (Lot No 819744 Vitrum AB Stockholm Sweden) with an activity of 24 8 U per mg was used as standard The insulin antibodies and ^{125}I insulin were supplied by the Radiochemical Centre Amersham England The assays were made in duplicates and the error of estimation was ± 5 per cent

At predetermined intervals from 1 day to 4 months the animals were sacrificed and specimens were taken from the pancreas for light and electron microscopic examinations The techniques used for morphological studies followed those of previous investigations (Boquist 1967 a and b) In addition a semi quantitative estimation of the pseudo isocyanin reaction (*Schiebler & Schiessler* 1959) was performed In "unknown" random sections (5 μ thick) from the pancreas of 10 zinc deficient and 10 control I hamsters the metachromatic reaction in the β cells of 5 islets was classified as — + ++ or +++

RESULTS

Tissue Concentration of Zinc

In Chinese hamsters fed the zinc deficient diet for 3 weeks the concentration of zinc in the testes was reduced as compared with the controls I (Table 1) No significant difference between the zinc deficient and control animals was found as to the concentration of zinc in heart kidneys liver and pancreas at that observation time

TABLE 1

Zinc Concentration in Various Tissues of Chinese Hamsters Fed Zinc Deficient or Control Diet for 3 Weeks

Tissues	Zinc deficient diet	Control diet 1
Heart	33 ± 2 (7)	37 ± 4 (7)
Kidneys	45 ± 2 (8)	48 ± 3 (8)
Liver	116 ± 2 (10)	108 + 3 (7)
Pancreas	85 ± 3 (8)	87 ± 3 (6)
Testes	97 ± 6 (8)	14 ± 3 (7)

The levels have been calculated as μg/g wet weight and represent mean values ± S.E.M The numbers of animals are given within brackets
Control diet 1 refer to standard laboratory diet

Urine Glucose

Glucosuria was not found in any of the zinc deficient or any of the 2 groups of control animals

Blood Glucose Level

Hyperglycaemic animals were not observed The fasting blood glucose values in the zinc deficient animals and in the 2 groups of control animals were all within the limits 102–121 mg/100 ml

TABLE 2

Intraperitoneal Glucose T[illegible]lerance of Chinese Hamst[illegible] [illegible] Zinc Def[illegible] [illegible] Diets f[illegible] Three We[illegible]

Diets	Time after administration of glucose: Minutes 0	1[illegible]	[illegible]0	Hours 1	2	4	6	8
Zinc deficient diet	105 ± 1.4 (28)	183 ± 3.4 (9)	219 [illegible] 1	[illegible]3[illegible] (11)	244 ± 6.7 [illegible]	105 ± 3.0 (10)	1[illegible] ± 3.4 (9)	115 ± 2.1 (1[illegible])
Control diet I	10[illegible] ± 2.[illegible] (10)	1[illegible] [illegible]	1[illegible] [illegible] 9	172 [illegible] 3.0 [illegible]	111 [illegible] 1.7 (9)	10[illegible] [illegible] (8)	110 ± 2.[illegible] (6)	10[illegible] ± 3.3 (6)
Control diet II	106 ± 2.4 (10	1[illegible]9 [illegible]	1[illegible] [illegible] 3.3 9	118 ± 3.1 (6)	103 ± 1.8 (9)	10[illegible] ± 2.9 (6)	104 ± 3.2 (6)	105 ± 3.3 (6)

The bl[illegible] glucose le[illegible] [illegible] mg 10[illegible] ml and represent mean values ± S.E.M.
[illegible] numbers of animals are given within brackets.
Control [illegible] refers to standard laboratory diet.
Control diet II refers to zinc deficient diet with the addition of zinc in water.

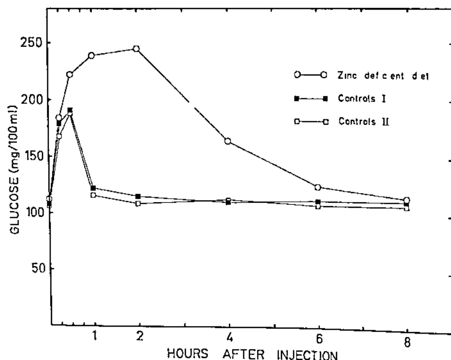


Fig 1

The intraperitoneal glucose tolerance of 3 groups of Chinese hamsters fed experimental diets for 3 weeks is shown Glucose at a concentration of 2 g/kg of body weight was injected intraperitoneally into 28 hamsters fed a zinc deficient diet 10 hamsters fed a standard laboratory diet (controls I) and 10 hamsters fed the zinc deficient diet with the addition of zinc (controls II) Blood glucose determinations were performed on a varying number of animals at 0 15 and 30 minutes as well as at 1 2 4 6 and 8 hours The heavy black lines denote the mean blood glucose levels for the 3 animal groups

Intraperitoneal Glucose Tolerance

In the hamsters fed the zinc deficient diet decreased glucose tolerance was observed already after this diet had been fed for one week Repeated tests at various time intervals showed persistent pathological values Though rather wide individual variations occurred no obvious tendency to a higher or lower degree of decreased glucose tolerance was present at repeated tests There was no difference between males and females nor were there any differences between younger and older animals The glucose tolerance curves for the controls I and II were rather similar and showed a peak value of about 180 mg/100 ml at 30 minutes and then a rather rapid return to the normal level In Table 2 and Fig 1 the results of the glucose tolerance tests on hamsters fed the experimental diets for 3 weeks are given

Intravenous Glucose Tolerance

Already when the diets had been fed for 1 and 2 days the blood glucose level at 30 minutes after the administration of glucose was increased in the zinc deficient animals as compared with the controls I. The values at 0 minute showed no difference between the animal groups. At 7, 14, 21, 3[illegible] and [illegible]8 days there was no difference between the blood glucose level of the animal groups at 0 minute whereas the level at 30 minutes was increased in the zinc deficient hamsters. It thus seemed that the intravenous glucose tolerance was decreased in the zinc deficient hamsters. As in the intraperitoneal tests no sex or age differences but rather wide individual variations of the blood glucose values were found. The results have been summarized in Table 3.

TABLE 3

Intravenous Glucose Tolerance of [illegible] Hamsters Fed Zinc Deficient [illegible] Diets

Diets	Time of feeding the experimental diets					
	1 [illegible] days		[illegible] 3 weeks		5 8 weeks	
	Minutes		Minutes		Minutes	
	0	[illegible]	0	30	0	30
Zinc deficient diet	10[illegible] [illegible] 2.9	[illegible]	10[illegible] ± [illegible]	[illegible]	107 ± 2.0	2[illegible] ± 5.1
	(13)		([illegible])		([illegible])	
Control diet I	10[illegible] ± 1.1	1[illegible]	[illegible]	[illegible]	107 ± 3.7	133 ± 3.9
	(7)		([illegible])		(4)	

The blood glucose levels at 0 and 30 minutes have been calculated as mg/100 ml and represent the mean values ± S.E.M. The numbers of the animals are given within brackets. Control diet I refers to standard laboratory diet.

TABLE 4

Serum Immunoreactive Insulin [illegible] Before and After (30 Minutes) Intravenous Glucose [illegible] in [illegible] Hamsters Fed Zinc Deficient [illegible]

Diets	Time of feeding the experimental diet					
	1 7 days		3 weeks		5 8 weeks	
	Minutes		Minutes		Minutes	
	0	30	0	30	0	30
Zinc deficient diet	3.1 ± 0.7	[illegible]1 ± 0.[illegible]	1.[illegible] [illegible] 0.6	1.9 ± 0.[illegible]	3.[illegible] ± 1.3	4.9 ± 1.7
	(13)		([illegible])		( )	
Control diet I	1.4 ± 0.5	2.5 ± 0.7	0.9 [illegible] 0.[illegible]	[illegible]0 [illegible] 0.2	2.6 ± 0.7	1.5 ± 0.7
	(7)		(7) p < 0.0[illegible]		(4)	

The concentrations have been calculated as [illegible] insulin/ml serum measured as ox insulin and represent the mean value ± S.E.M. Where the difference between the value at 0 and 30 minutes within one time and dietary group is statistically significant the p value is included. The numbers of the animals are given within brackets. Control diet I refers to standard laboratory diet.

Serum Immunoreactive Insulin

The results of the serum insulin determinations before and 30 minutes after the intravenous administration of glucose have been summarized in Table 4 In the zinc deficient hamsters there was no statistically significant difference between the values at 0 and 30 minutes in any of the 3 time groups The insulin value at 30 minutes was higher than at 0 minute in the hamsters fed the control diet I for 2–3 weeks In the two other time groups of the controls I there was no statistically significant difference between the values at 0 and 30 minutes As to the serum insulin concentration before the administration of glucose there was no statistically significant difference between the animal groups

Light Microscopic Findings

The general appearance of the islets was normal No infiltration of inflammatory cells was present Decreased granulation of the β cells occurred rather often in the zinc deficient animals The sulphide silver method for light microscopic study of heavy metals was positive in the central and peripheral region of the islets both in zinc deficient and control hamsters Though the positive reaction of this method some

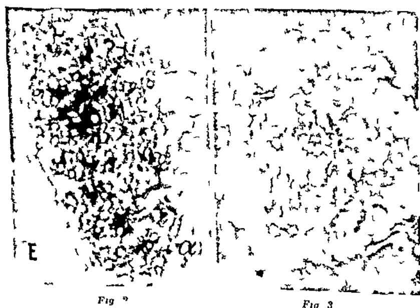


Fig 2 Pancreatic islet of a Chinese hamster fed control diet I for 2 weeks showing metachromatic reaction with pseudoisocyanin in the central β-cells (β) The peripheral α-cells (α) and the exocrine parenchyma (E) are unaffected Bouin s fixative Pseudo isocyanin procedure × 200

Fig 3 Pancreatic islet of a Chinese hamster fed zinc deficient diet for 2 weeks showing almost no metachromatic reaction with pseudo isocyanin Bouin s fixative Pseudo isocyanin procedure × 300

Intravenous Glucose Tolerance

Already when the diets had been fed for 1 and 2 days the blood glucose level at 30 minutes after the administration of glucose was increased in the zinc deficient animals as compared with the controls. The values at 0 minute showed no difference between the animal groups. At 7, 14, 21, 35 and 58 days there was no difference between the blood glucose level of the animal groups at 0 minute, whereas the level at 30 minutes was increased in the zinc deficient hamsters. It thus seemed that the intravenous glucose tolerance was decreased in the zinc deficient hamsters. As in the intraperitoneal tests no sex or age differences but rather wide individual variations of the blood glucose values were found. The results have been summarized in Table 3.

TABLE 3

Intravenous Glucose Tolerance of Chinese Hamsters Fed Zinc Deficient or Control Diets

Diets	Time of feeding the experimental diets					
	1–7 days		2–3 weeks		5–8 weeks	
	Minutes		Minutes		Minutes	
	0	30	0	30	0	30
Zinc deficient diet	108 ± 2 9	[illegible]77 ± 4 2	10[illegible] ± 2 4	[illegible]18 ± 5 7	107 ± 2 0	2[illegible]1 ± 3 1
	(13)		(9)		(7)	
Control diet I	106 ± 1 4	180 ± 2 1	109 ± 2 1	177 ± 2 3	107 ± 3 7	183 ± 3 9
	(7)		(7)		(4)	

The blood glucose levels at 0 and 30 minutes have been calculated as mg/100 ml and represent the mean values ± S.E.M. The numbers of the animals are given within brackets. Control diet I refers to standard laboratory diet.

TABLE 4

Serum Immunoreactive Insulin Level before (0 Minute) and after (30 Minutes) Intravenous Glucose Administration in Chinese Hamsters Fed Zinc Deficient or Control Diet

Diets	Time of feeding the experimental diets					
	1–7 days		2–3 weeks		5–8 weeks	
	Minutes		Minutes		Minutes	
	0	30	0	30	0	30
Zinc deficient diet	3 1 ± 0 7	2 1 ± 0 6	1 8 ± 0 6	1 9 ± 0 2	3 2 ± 1 3	4 9 ± 1 7
	(13)		(9)		(7)	
Control diet I	1 4 ± 0	2 5 ± 0 7	0 9 ± 0 2	2 0 ± 0 2	2 6 ± 0 7	1 5 ± 0 7
	(7)		(7) $p < 0.05$		(4)	

The concentrations have been calculated as ng insulin/ml serum measured as ox insulin and represent the mean values ± S.E.M. Where the difference between the value at 0 and 30 minutes within one time and dietary group is statistically significant the p value is included. The numbers of the animals are given within brackets. Control diet I refers to standard laboratory diet.

times was weaker in the zinc deficient animals this did not allow any estimation of the zinc content in the islets not even semi quantitatively No degenerative changes of the β cells were recorded The α_1- and α cells were unaffected

The pseudo isocyanin procedure for insulin was often negative in the zinc deficient animals as compared with the controls (Figs 2 and 3) The semi quantitative estimation of the results of the pseudo isocyanin procedure gave the following results

	—	+	++	+++
Zinc deficient hamsters	3	4	2	1
Control I hamsters	0	2	2	6

Electron Microscopic Findings

The β cells of the zinc deficient animals often showed well developed endoplasmic reticulum and Golgi complex as well as degranulation (Fig 4) The other organelles were unaffected in these cells Th α_1 α and agranular cells were unchanged in the zinc deficient animals In the control hamsters no ultrastructural alterations were recorded

DISCUSSION

In rats fed the same zinc deficient diet and given the same water as that used in the present study clear signs of zinc deficiency appeared after 3 weeks (*Bergman et al* 1968) The Chinese hamsters fed the zinc deficient diet showed decreased zinc concentration in testes but not in heart liver kidneys and pancreas This conforms to findings in zinc deficient rats where the concentration of zinc is markedly reduced in testes and bones but not in other tissues (*Macapinlac et al* 1966) Thus it seems that the zinc deficient diet used in the present work was effective This is of value to know as it is known that a great risk of contamination by zinc is involved in biological experiments (*Anonymous* 1968)

In the urine and blood glucose determinations as well as in the intraperitoneal glucose tolerance tests where two kinds of control diets were used any difference between the effects of these diets was not found Because of this the use of only one kind of control diet for the atomic absorption spectrophotometry the intravenous glucose tolerance tests and the serum insulin determinations seems to be justified

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Fig 4

Portion of pancreatic islet of a Chinese hamster fed zinc deficient diet for 1 week demonstrating two α_2-cells (α) and one agranular cell (γ) without alterations In most of the β-cells (β) the occurrence of secretory granules is rather sparse In one of the β-cells there is a cilium (Ci) protruding into an intercellular space × 4 000

Zinc deficient rats exhibit anorexia and reduced feed efficiency (*Prasad et al* 1967) It has also been found that the glucose tolerance curve of control rats fed restricted diets is low and flat as compared with that of zinc deficient rats (*Macapinlac et al* 1966) Since pathological changes including elevated glucose tolerance curves appeared in Chinese hamsters fed zinc deficient diet but not in those fed standard laboratory diets or zinc deficient diets with the addition of zinc it seems to be more probable that the zinc deficiency *per se* is the cause of these effects and that possible differences in dietary intake play a subordinate role The fact that no age or sex differences were found in the present study seems also to support the view that deficiency of zinc was responsible for the pathological alterations and it indicates that no age or sex factors interfered with the blood glucose as in rats (*Hajdu & Rona* 1967 *Klimas* 1968) As to the plasma zinc concentration in man it is known that sex and age differences are insignificant (*Davies et al* 1968)

Since there were neither hyperglycaemia or glucosuria nor any marked morphological islet lesions in the Chinese hamsters fed the zinc deficient diet, it is obvious that the frequency of spontaneously occurring overt diabetes mellitus in this species could not be increased by feeding such a diet at least not with the present observation times On the other hand the occurrence of decreased glucose tolerance in these hamsters might indicate that zinc deficiency evoked a pre diabetic state Similarly decreased glucose tolerance was found in the Chinese hamster also after pancreatectomy (*Boquist* 1967c) and after the administration of alloxan (*Boquist* 1968)

Hove et al (1937) observed that the oral glucose tolerance curves were irregular and delayed in zinc deficient rats whereas the glucose level and liver glycogen were normal This was interpreted as indicative of impaired absorption of glucose from the bowel In the Chinese hamsters fed the zinc deficient diet both the intraperitoneal and intravenous glucose tolerance was impaired Thus it seems that the absorption of glucose from the peritoneal cavity is not conspicuously altered in these animals As a consequence of this the intraperitoneal glucose tolerance tests were considered to be representative

On the basis of a study of 4 pairs of zinc deficient and control rats *Quarterman et al* (1966) have stated that the zinc deficient animals had decreased concentration of plasma insulin These authors also suggested that the rate of secretion of insulin in zinc deficient rats was decreased in response to glucose stimulus In the present study there was no significant difference in the serum insulin levels in zinc deficient and control animals and the values conform to those found by *Gerritsen & Dulin* (1967) in nonketotic non diabetic Chinese hamsters The interpretation of the responses of the serum insulin to intravenous glucose administration is somewhat difficult No difference between the values at 0 and 30 minutes was observed in the zinc deficient hamsters

seemingly indicating that there is no such response in these animals. In the control hamsters, on the other hand, the values at 0 and 30 minutes were found to differ in one of the three time groups, whereas no difference occurred in the other two groups. The cause of these equivocal findings is still unknown and will be further analysed in a forthcoming work.

The plasma zinc concentration in man has been said to show only small variations normally (*Davies et al.* 1968), possibly because of an efficient homeostatic mechanism for zinc (*Anonymous* 1968). Oral or intravenous glucose load in man induces a rapid fall of the plasma zinc level, followed by a rather rapid recovery (*Davies et al.* 1968). In pregnant female rats and in growing male rats *Dreosti et al.* (1968) observed a fall of the plasma zinc concentration already after a zinc deficient diet had been fed for one day. After that time the plasma zinc decrease became progressively less marked and a plateau was reached by about 7 days. This was suggested to be due to the *inability of these rats to mobilize their body deposits adequately to accomodate for even a short period of dietary zinc restriction. The glucose tolerance tests in the present study show that also the Chinese hamster may react rapidly to dietary zinc deficiency and that there are no marked alterations in short or long time experiments. The cause of the impaired glucose tolerance of the zinc deficient Chinese hamsters is not known. Though there was no decrease of the serum insulin concentration in these animals, the light and electron microscopic examinations indicate that the β-cells had a decreased granulation and thus possibly also a decreased amount of insulin. This possibility is supported by the results *of the semi-quantitative estimation of the pseudo-isocyanin *procedure*. Though the light microscopic sulphide silver reaction did *not allow* any quantitative metal estimation and though the atomic absorption spectrophotometry did not reveal any decreased concentration of zinc in the pancreas, it seems, however, possible that there might be a decreased content of zinc in the islets of the zinc deficient animals. The ultrastructural finding by *Pihl & Falkmer* (1967) of a decreased amount of sulphide silver positive secretion granules in the β cells of zinc deficient rats may give some support to this view. If the concentration of zinc in the islets is diminished and if it is accepted that zinc is concerned with the storage of insulin in the β cells, it may be speculated that the amount of stored insulin is decreased in zinc deficient animals. When excess glucose is administered to these animals, as in glucose tolerance tests, an insufficient amount of insulin may be available, resulting in pathological effects on the glucose tolerance curves. When glucose is not given in excess to the animals, the production and secretion of insulin seem to be sufficient and no hyperglycaemia or glucosuria is observed. The lack of insulin response to glucose in the zinc deficient animals and the occurrence of this response in some of the control hamsters might support these speculations. As it is known that a de-

creased tissue concentration of zinc in zinc deficient rats is combined with decreased activities of certain enzymes (*Prasad et al* 1967) the effect of zinc deficiency in the Chinese hamster may also be thought to be due to enzymatic derangement Further studies are required to test the validity of these speculations

SUMMARY

Three groups of Chinese hamsters were fed a zinc deficient diet a standard laboratory diet and a zinc deficient diet with the addition of zinc in water respectively By atomic absorption spectrophotometry the concentration of zinc was determined in samples from heart kidneys liver pancreas and testes of animals fed the experimental diets for 3 weeks A decreased concentration of zinc was found in the testes indicating that there actually was an effect of the zinc deficient diet

Glucosuria or hyperglycaemia could not be elicited by feeding the zinc deficient diet The intraperitoneal and intravenous glucose tolerance was impaired in hamsters fed the zinc deficient diet The serum immunoreactive insulin level in zinc deficient hamsters did not deviate from that in the control animals In hamsters fed the zinc deficient diet no significant increase of this level occurred after glucose injection

In the light and electron microscopes decreased granulation of the islet β cells of zinc deficient hamsters was observed The pseudo isocyanin method for insulin disclosed weaker reaction in the β-cells of animals fed the zinc deficient diet The α_1 α and agranular cells showed no morphological alterations

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GRANULOMATOUS HYPOPHYSITIS AND THYROIDITIS WITH LYMPHOCYTIC ADRENALITIS

By

WILLIAM KIÆR and J O RYTTER NØRGAARD

Received 10 xii 68

In rare cases the pituitary gland of elderly women may show characteristic granulomas not only with lymphocytic infiltration and epithelioid cells but also with true giant cells Independently of the granulomas giant cells are present also in intact glandular tissue The granulomas are reminiscent partly of miliary tubercles but are entirely unrelated to tuberculosis or to syphilis (Simmonds 1917) *Sheehan & Summers* in 1949 collected 18 such cases including Simmonds 4 as pituitary giant cell granulomas They concluded that the group may include lesions due to a number of different causes such as syphilis tuberculosis or sarcoidosis but that in the majority of cases they appear to represent a specific disease process whose aetiology is unknown

It is our impression from the recent literature that the granulomatous pituitary lesions with giant cells may be divided into 3 groups

(1) Granulomas due to tuberculosis syphilis or sarcoidosis
(2) Granulomas of unknown aetiology with secondary changes in other organs especially the thyroid and adrenals as a consequence of pituitary insufficiency—or without such secondary lesions if only a minor part of the hypophysis is involved
(3) Granulomas of unknown aetiology combined with changes in other organs especially the thyroid and adrenals which can hardly be secondary to pituitary insufficiency mainly because the histological appearances in these organs are of a type entirely different from that of changes caused by impaired pituitary function

The object of the present paper is to report and discuss a case of the last mentioned group

William Kiær Prosektor dr med Svendborg Amts Patologiske Institut Centralsygehuset Svendborg Denmark

CASE REPORT

A 74 year old woman was *admitted* with the diagnoses Vertigo cerebral arteriosclerosis and arteriosclerotic heart disease

Ten years previously she had been admitted with concussion and fracture of the cervical spine No signs of *fracture of the skull* During the entire intervening period the patient had been suffering from frequent bouts of *dizziness headache* and tinnitus

Gynaecological history One normal delivery at the age of 35 Menopause in the 40 ies

No tuberculosis in her environment

Present illness During the past 6 months the patient had been off colour and sensitive to cold Three months *prior to the present admission* influenza like symptoms simultaneously with others *in her environment* Ever since she had been suffering from bouts of dizziness increasing headache tinnitus and impaired hearing No tendency to fall no nausea or vomiting She also complained of tenderness in all joints said to *have arisen after* the influenza Appetite poor but she did not know whether she had lost weight During the past 3–4 weeks she had been in bed The dizziness had been treated with thiethylperazin malcate (Torecan) and ergotamine tartrate *(Bellergal)* tablets but ineffectively

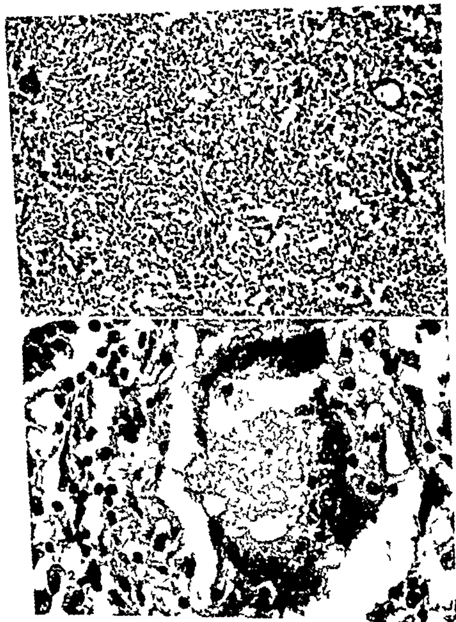
Physical examination Stolid *and lethargic* with dull and slow reactions Skin dry and scaly Face somewhat congested Nutritional condition considerably above medium *Not in shock* *BP 170/70* pulse rate 60 temperature 37 0 C No visible goitre No *neurological* signs Achilles reflexes absent Other reflexes normal

Laboratory findings ESR 33 67 mm/hour Hb 74–85 *per cent Red cells* 3 1f mill/µl White cells 2100 5700/µl Differential count Relative lymphocytosis Platelets 239 000/µl Total protein 6 6–7 4 g/100 ml Paper electrophoresis 23 per cent γ globulin (normal 10 18 per cent) and 59 per cent albumin (*normal* 60–70 per cent) Other fractions within the normal range Serum electrolytes Potassium 4 1 3 0 mEq/l sodium 137–146 mEq/l chloride 90 97 mEq/l and total CO 19–2° mEq/l Serum calcium 9 3 mg/100 ml serum phosphorus 4 9 mg/100 ml PBI 2 1 and 2.3 µg/100 ml T test 6 1 and 5 9 per cent Serum cholesterol 204 250 *mg/100 ml* Blood sugar 71–80 mg/100 ml Antistreptococcal hyaluronidase agglutination reaction with haemolytic streptococci as antigen latex fixation test *anti human* globulin consumption test anti nuclear factor LE cells on three occasions all negative Test for cytoplasmic thyroid antibody negative *Antistreptolysin titre* 200 unit ECG Auricular fibrillation (slow perpetua) Low voltage Ears and eyes No abnormalities EEG Severely abnormal with runs of 2–3 cps *activity of* increased amplitude especially bitemporally Generally reduced dominant frequency

Owing to the patient s appearance and lethargy myxoedema soon suggested itself and the patient was put on thyroid medication 60 mg daily However this had no particular effect upon the clinical condition One week after the institution of the treatment she developed fever vomiting and diarrhoea and went into shock She was treated with fluid oxygen digitalis (Cedilanid) and terramycin A sample of faeces sent in for culture of pathogenic intestinal bacteria showed *growth of Staph* aureus The condition rapidly deteriorated and the patient died within 24 hours Throughout the stay in hospital she had been hypotensive *having a blood pressure* ranging from 70/50 to 130/90 without having been in clinical shock until terminally

Autopsy (5730) Slight diffuse enlargement of the *thyroid* which was of uniform firm consistency and greyish red in colour Adrenals pancreas and pituitary grossly normal No lymph node enlargement Bronchopneumonic infiltrations in the lower lobes of both lungs On microscopic examination changes were found in the pituitary *thyroid* and adrenals

The *pituitary* (Figs 1 and 2) showed very severe changes throughout the anterior lobe There were numerous medium sized to large round or irregular partly confluent granulomas built up of plump epithelioid cells Neither the cells nor the granulomas were as well defined as in



Figs 1 2

Fig 1 Pituitary gland Ill defined confluent epithelioid cell granulomas Giant cells of varying size and appearance Lymphocytic infiltration × 175 Haematoxylin eosin

Fig 2 Pituitary gland Vacuolized giant cell with marginal irregularly distributed nuclei of varying size × 500 Haematoxylin eosin

CASE REPORT

A 74 year old woman was admitted with the diagnoses Vertigo, cerebral arteriosclerosis and arteriosclerotic heart disease.

Ten years previously she had been admitted with concussion and fracture of the cervical spine. No signs of *fracture of the skull*. During the entire intervening period the patient had been suffering from frequent bouts of dizziness, headache and tinnitus.

Gynaecological history. One normal delivery at the age of 35. Menopause in the 40 ies.

No tuberculosis in her environment.

Present illness. During the past 6 months the patient had been off colour and sensitive to cold. Three months prior to the present admission influenza like symptoms simultaneously with others in her environment. Ever since she had been suffering from bouts of dizziness, increasing headache, tinnitus and impaired hearing. No tendency to fall, no nausea or vomiting. She also complained of tenderness in all joints, said to have arisen after the influenza. Appetite poor, but she did not know whether she had lost weight. During the past 3–4 weeks she had been in bed. The dizziness had been treated with thiethylperazine maleate (Torecan) and ergotamine tartrate (Bellergal) tablets but ineffectively.

Physical examination. Stolid and lethargic, with dull and slow reactions. Skin dry and scaly. Face somewhat congested. Nutritional condition considerably above medium. Not in shock. BP 120/70, pulse rate 60, temperature 37.0 C. No visible goitre. No neurological signs. Achilles reflexes absent. Other reflexes normal.

Laboratory findings. ESR 33/67 mm/hour. Hb 71.8% per cent. Red cells 3.16 mill/μl. White cells 3100–5200/μl. Differential count: Relative lymphocytosis. Platelets 239 000/μl. Total protein 6.6–7.4 g/100 ml. Paper electrophoresis: 23 per cent γ globulin (normal 10–18 per cent) and 50 per cent albumin (normal 60–70 per cent). Other fractions within the normal range. Serum electrolytes: Potassium 4.1–3.0 mEq/l, sodium 137–146 mEq/l, chloride 90–97 mEq/l and total CO_2 19–32 mEq/l. Serum calcium 9.3 mg/100 ml, serum phosphorus 4.9 mg/100 ml, PBI 9.1 and 2.3 μg/100 ml. T₃ test 6.1 and 5.9 per cent. Serum cholesterol 204–250 mg/100 ml. Blood sugar 71–80 mg/100 ml. Antistreptococcal haemolysin, antistreptolysin agglutination reaction with haemolytic streptococci as antigen, latex fixation test, anti human globulin consumption test, anti nuclear factor, LE cells on three occasions: all negative. Test for cytoplasmic thyroid antibody negative. Antistreptolysin titre 200 units. ECG: Auricular fibrillation, low perpetual T, low voltage. Ears and eyes: No abnormalities. EEG: Severely abnormal with runs of 2–3 cps activity of increased amplitude, especially bitemporally. Centrally reduced dominant frequency.

Owing to the patient's appearance and lethargy, myxoedema soon suggested itself and the patient was put on thyroid medication (60 mg daily). However, this had no particular effect upon the clinical condition. One week after the institution of the treatment she developed fever, vomiting and diarrhoea and went into shock. She was treated with fluid, oxygen, digitalis (Cedilanid) and terramycin. A sample of faeces sent in for culture of pathogenic intestinal bacteria showed growth of Staph. aureus. The condition rapidly deteriorated and the patient died within 24 hours. Throughout the stay in hospital she had been hypotensive, having a blood pressure ranging from 70/50 to 130/90 without having been in clinical shock until terminally.

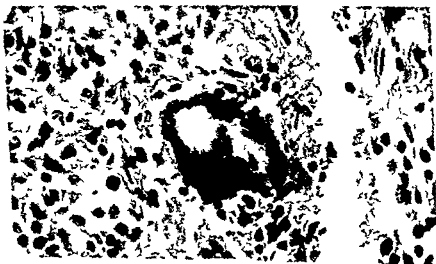
Autopsy (5730). Slight diffuse enlargement of the thyroid, which was of uniform firm consistency and greyish red in colour. Adrenals, pancreas and pituitary grossly normal. No lymph node enlargement. Bronchopneumonic infiltrations in the lower lobes of both lungs. On microscopic examination changes were found in the pituitary, thyroid and adrenals.

The *pituitary* (Figs. 1 and 2) showed very severe changes throughout the anterior lobe. There were numerous medium sized to large round or irregular, partly confluent granulomas built up of plump epithelioid cells. Neither the cells nor the granulomas were as well defined as in



Fig 4

Thyroid gland Granulomatous area Vacuolized giant cell Lymphocytic infiltration × [illegible] Haematoxylin-eosin



Fig

Thyroid gland Giant cell with irregularly distributed partly marginal nuclei of varying size × 500 Haematoxylin eosin

was preserved the follicles were on the whole small with sparse colloid and more or less enlarged epithelial cells with eosinophilic cytoplasm Here and there granulomas corresponding to those found in the pituitary both in respect to giant cells and epithelioid cell granulomas (Figs 4 and 5)

Case summary Autopsy on a 74 year old woman who had exhibited signs of pluriglandular insufficiency revealed in the pituitary giant cell granulomas, in the thyroid goitre showing partly granulomas of the same type and partly changes as in Hashimoto's thyroiditis while the adrenals exhibited lymphocytic infiltration. Histological examination of the lymph nodes, skin, subcutaneous tissue, brain tissue, leptomeninges, myocardium, liver, spleen and kidneys showed no abnormalities.

DISCUSSION

There is no evidence to indicate that the pathological changes of the pituitary gland were caused by tuberculosis, sarcoidosis or syphilis. Owing to the localization the named diseases would be expected to have been generalized. In addition sarcoidosis involving the pituitary will in more than 90 per cent give rise to diabetes insipidus with involvement of the posterior lobe, syphilis usually affects the entire pituitary and tuberculosis seldom causes hypopituitarism (16). Furthermore the morphological similarity to the three diseases is fairly modest. In our case the giant cells were of extremely varying size and nuclearity, partly with irregularly distributed nuclei and in places with coarsely vacuolized cytoplasm. The epithelioid cells were strikingly plump and the granulomas ill defined. We found no hyalinosis, fibrosis, caseous necroses or gumma formation. The histological appearances corresponded accurately to previously described cases of pituitary giant cell granulomas of unknown aetiology.

Sheehan & Summers (1949) reported two cases of chronic fibrous lesions in the pituitary gland which they interpreted as sequelae to cranial injury, in particular with fracture of the base of the skull. These authors suggested that there might be a question of healed granulomas. Our patient had a history of cranial injury 10 years before her death, but it seems unlikely that this trauma can be related to the active granulomatous process which was entirely devoid of fibrosis.

It is not likely either that the appreciable changes in the adrenals could have been secondary to pituitary insufficiency. True, the patient had reduced function of the anterior pituitary lobe. This is indicated by the clinical findings as well as by the fact that more than three quarters of the anterior lobe had undergone destruction (21). But apart from doubtful narrowing of the adrenal cortex, there was well marked lymphocytic infiltration which does not accord with the usual adrenal reaction to hypopituitarism. On the basis of the clinical signs as well as the histological appearances it must be considered likely that the entire disease ran a fairly brief course so that the usual secondary adrenal changes did not have time to develop. It is more difficult to appraise the thyroid changes in relation to pituitary giant cell granulomas, but they differed in essential respects from the atrophy, fibrosis

and lymphocytic infiltration often seen secondary to reduced pituitary function In our case there was mild goitre which presented itself mainly as Hashimoto s thyroiditis but here and there the thyroid showed changes identical with those found in the pituitary and entirely unlike de Quervain s granulomatous thyroiditis

It is worth considering whether this case may have represented an extended Schmidt syndrome involving the pituitary *Schmidt*, in 1926 described two patients with hypofunction of the adrenals as well as of the pituitary gland The adrenals showed lymphocytic infiltration and atrophy as in idiopathic Addison s disease and the thyroid changes reminiscent of Hashimoto s thyroiditis Later *Bloodworth et al* (1954) found signs of hypothyroidism in 13 out of 35 patients with primary adrenal insufficiency *Kracht & Hachmeister* (1966) interprete Schmidt s syndrome as the link between auto immune thyroiditis and those types of primary adrenocortical atrophy which have been considered an auto immune disease *Nerup et al* (1966) found antibody to cytoplasmic antigen in the adrenal cortex of 31 out of 48 patients with "idiopathic Addison s disease Of these 31 patients 21 also had thyroid antibodies *Bl ard et al* (1967) demonstrated the same antibody combination in 12 out of 64 patients with adrenal insufficiency They pointed out that among 57 patients with Hashimoto s disease none had adrenal antibodies and that the incidence of Addison s disease in patients with primary myxoedema is low Therefore the autoimmune processes which affect the two glands appear to represent a distinct disease entity and this is further supported by the fact that the antibodies are organ specific We have no proof that our case might be a triglandular Schmidt s syndrome with auto immune processes directed against all three glands but below we shall review certain morphological studies which have given rise to reflections in this direction

Goudie & Pinkerton (1962) suggested the possibility of auto immunization in a case of hypophysitis and thyroiditis The thyroid changes were reminiscent of ours consisting in Hashimoto s thyroiditis with giant cells but the hypophysis showed severe lymphocytic infiltration without giant cells The adrenals could not be found *Hume & Roberts* (1967) have adduced similar suggestions on the basis of a case of lymphocytic hypophysitis and thyroiditis (without giant cells) combined with adrenal atrophy and pernicious anaemia *Oelbaum & Wainwright* (1950) reported a case of typical giant cell granulomas associated with multiple granulomas of the same type in the adrenals which were moreover atrophic The thyroid exhibited atrophy fibrosis and lymphocytic infiltration These authors did not further discuss the aetiology but stated that the degree of atrophy in the secondary endocrine organs was not of the advanced type seen following long lasting hypopituitarism This was in keeping with the clinical biochemical and histological studies which indicated that the pituitary lesion was subacute About the same applies to our case only more so

Doniach & Wright (1951) in one of their two cases of pituitary giant cell granulomas found granulomas of exactly the same histological architecture in the adrenals which also showed atrophy. The thyroid exhibited only adenomata. These authors too did not enter into any aetiological details. *Morgenstern* (1961) considered the possibility of autoimmunization in a patient with pituitary giant cell granulomas combined with granulomatous thyroiditis. He also found numerous granulomas in the myocardium and a few in the kidneys and adrenals. *Blersch & Robbins* (1952) published 4 cases of giant cell granulomas. Three were presumably due to sarcoidosis but this could be ruled out in the fourth case. The adrenals were atrophic with severe diffuse lymphocytic infiltration. The thyroid gland was not studied. *Subbaswamy et al.* (1967) reported one case of pituitary giant cell granulomas in which the thyroid showed granulomas of the same type. The adrenals were grossly normal but nothing is stated concerning histological findings. Since the pituitary changes were slight and involved only a small part of the gland these authors did not interpret the thyroid lesions as being secondary to the pituitary giant cell granulomas feeling that the pathological appearances were more likely to be due to a common agent of unknown nature.

All the reported cases of pituitary giant cell granulomas have had only deficient or no serological studies with a view to autoimmunization. In our case the antinuclear factor, antihuman globulin consumption test, and study for cytoplasmic thyroid antibody by immune fluorescence technique were negative. The last mentioned test is positive in about 90 per cent of sera from patients with diffuse chronic thyroiditis of the Hashimoto type and in about two thirds of sera from patients having acquired myxoedema without goitre (*Halberg* 1967). This would seem to indicate that autoimmune thyroiditis was rather unlikely in our patient. Moreover *Goudie* (1968) and *Nerup et al.* (11) did not find specific pituitary antibodies in patients with idiopathic panhypopituitarism but it is not known whether these materials included cases of pituitary giant cell granulomas. There is the possibility that the present technique is not able to demonstrate antibodies in pituitary giant cell granulomas. *Goudie* (1962, 1968) feels that the purely lymphocytic type of hypophysitis is presumably of autoimmune nature, and this assumption is supported by experimental investigations (11), while pituitary giant cell granuloma is perhaps an entirely different disease and that at present it is not possible to decide its nature. He believes there is a possibility that pituitary giant cell granuloma is an autoimmune disease in which the morphological signs of the immune reaction differ considerably from that found in most other autoimmune diseases. He goes on: *I should however say that granulomata are found in the liver in primary biliary cirrhosis which certainly is associated with non organ specific*

case of idiopathic adrenal atrophy in which strong adrenal auto antibody was definitely present and in which a single sarcoid like follicle was present in the small amount of adrenal cortex which survived (7) Our case had granulomatous hypophysitis and thyroiditis but also thyroid and adrenal changes of the type seen in auto immunization The thyroid and adrenal changes cannot be interpreted as a consequence of pituitary insufficiency but all three lesions are presumably concurrent

SUMMARY

A case of hypophysitis thyroiditis and adrenalitis in a patient with signs of insufficiency of the anterior pituitary lobe is reported The pituitary changes were typical of the granulomatous hypophysitis with giant cells The thyroid gland exhibited the same histological appearances and besides lesions like those seen in Hashimoto s thyroiditis In the adrenals lymphocytic infiltration was present It is considered likely that the disease has been rather short lasting and that the named organic changes are secondary to the same action which is un known but the possibility of auto immunization is ventilated

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ACTINOMYCES IN TONSILLAR TISSUE

A Histological Study of a Tonsillectomy Material

By

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Received 8 xi 68

Actinomyces as the cause of human disease has been known for 90 years Its habitat is the oral cavity It is frequently found in carious teeth dental calculus (tartar) and occasionally in the tonsils

Actinomycosis may affect any organ or tissue More than 50 per cent of the cases however are located to the face and neck Among out patients the cervicofacial form is even more predominant

Actinomyces is considered as an opportunistic microbe (*McKinnon* 1962 *Peabody & Seabury* 1960 *Utz* 1962) *Israel* (1885) held the oral cavity to be the chief reservoir *Ruge* (1885) found the granules in ton sillar crypts but did not believe them to be true *Actinomyces* colonies Lord (1910) and *Emmons* (1938) isolated *Actinomyces* from tonsillar tissue *Emmons* found the hyphae by direct examination in 74 of 200 pairs of tonsils (37 per cent) and by culture in 14 per cent in a New York material *Wilkinson* (1929) examined the tonsils of 10 000 cases of tonsillectomy performed at the Mayo Clinic 1923–26 By histological examination he found granules resembling *Actinomyces* in 177 cases (1 8 per cent)

The frequencies observed by *Wilkinson* and by *Emmons* differ con siderably Information on *Actinomyces* granules according to age and sex is scarce Further study therefore seemed warranted

MATERIAL AND METHODS

During 1967 tonsils from 103 patients were received in the Department of Pathology Ullevål Hospital The material came from the Department of Oto Rhino Laryngology In two cases only one tonsil was extirpated

The indications for removal are given in Table 1

For routine diagnosis the tonsils were fixed in 4 per cent neutral formaldehyde solution for about 24 hours Each tonsil was transsected and a central slice em bedded in paraffin Two sections about five microns thick were stained with haem toxylin and eosin (H E)

The sections have been re-examined for *Actinomyces* colonies In a number of cases the preliminary diagnosis was doubtful In these cases and in all cases were a tentative positive diagnosis had been given new sections were cut and stained with Gram's stain and with periodic acid *Schiff* (PAS)



Fig 1

Actinomyces colonies in tonsillar tissue H E stain Magnification × 75

Fig 2

The ray fungus with the *hyphae* arranged as spokes in a wheel Gram's stain Magnification × 500

TABLE 1
Indications for Tonsillectomy Number of Cases

Chronic tonsillitis	86
Chronic tonsillitis and adenoids	12
Glomerulonephritis	2
Peritonsillar abscess	2
Diagnostic in brain tumour (craniopharyngeoma)	1
Total	103

Identification

The typical picture of an *Actinomyces* colony is that of the ray fungus. The branching bacterial bodies—*hyphae*—are intermingled and arranged like spokes in a wheel (Figs. 1 and 2). In good sections with no shrinkage the clublike ends of the *hyphae* can be seen in the periphery of the colonies.

Actinomyces is a Gram positive organism. Central portions of large colonies are sometimes Gram negative. In H.E. sections the colonies are dark with a slight bluish tint. Like most microfungi *Actinomyces* takes the PAS stain which makes the colonies stand out conspicuously so that they are easily recognized.

RESULTS

In all cases cellular debris and bacterial colonies were found in the crypts. *Actinomyces* colonies were found in 17 of the 103 cases. This is a minimum number as serial sectioning has not been carried out. The age and sex distribution is given in Table 2. Uncertain colonies (numbers in brackets) either had a doubtful *Gram* reaction or seemed to be broken up by a crushing trauma during extirpation. Some small colonies did not show the typical picture of spokes in a wheel.

TABLE 2
Actinomyces Colonies in 103 Consecutive Tonsillectomy Cases According to Age and Sex. Doubtful Colonies within Brackets

Age interval	Males		Females		Total	
in years	Cases	Colonies	Cases	Colonies	Cases	Colonies
Under 5	8	1	4	0	12	1
5– 9	9	1	5	0	14	1
10–14	9	1	8	1 (1)	17	2 (1)
15–19	10	1 (1)	26	7 (2)	36	8 (3)
20–24	6	1	13	3 (1)	19	4 (1)
25–37	4	0 (1)	1	1	5	1 (1)
Total	46	5 (2)	57	12 (4)	103	17 (6)

An increasing number of colonies are found after the age of 15 (Table 3). This increase does not quite attain significance at the conventional level of 5 per cent probability.

In 101 of the cases the histological diagnosis was chronic tonsillitis /

hyperplasia of tonsillar tissue. In one case the tonsillitis was subacute and in another an abscess wall was found.

The *Actinomyces* granules are easily overlooked by routine examination. In none of the 17 cases were the colonies recognized at the first examination. In 6 of them, however, "fungus colonies" were described

TABLE 3
Actinomyces Colonies before and after 15 Years

Age group	Colonies	No colonies	Total
14 – under	4 (9.3%)	39	43
15 – over	13 (21.7%)	47	60
Total	17 (16.6%)	86	103

P = 0.08 (Fisher-Irving test)

DISCUSSION

Actinomyces very seldom affects the lymphatic system (*Cope* 1938). It is customary to regard the *Actinomyces* granules found in tonsils as mere saprophytes. It should be obvious, however, that neither the present material nor any other tonsillectomy material reported in the literature can be considered normal. The possibility that the granules may be connected with chronic tonsillitis cannot be excluded without consideration.

In *Williamson*'s material collected in the Mid-Twenties, the age and sex distribution is not given in detail. Although his method of examination hardly differs from the present, it is therefore somewhat doubtful to compare it with the present material. The difference in frequencies, however, would statistically be found highly significant ($\chi^2 = 115 > \chi^2_{.001} = 10.827$).

Emmons (1938) cut the tonsils in thin slices and examined the contents of the crypts by direct examination. In this way he found *Actinomyces* in 37 per cent of the cases. He admits the possibility, however, that some of the filamentous organism seen and listed as *Actinomyces* were not correctly identified and thinks this may partly explain why he only found *Actinomyces* by culture in 17 per cent of the cases. His material can, because of the difference in methods and aim, hardly be compared with the present material.

Williamson (1929) on the other hand looked for granules and the difference between his and the present material requires an explanation. 1. Either *Williamson* set up far more strict criteria for what he considered to be colonies. According to his illustrations this is hardly the case. 2. Or the proportion of children was higher. Although the number of children in which granules were found is not stated, the distribution in the total material is much the same. 3. Due consideration should

also be given to the possibility that he may just have overlooked the colonies but 4 The fact that indications for tonsillectomy are much stricter now than four decades ago may offer the explanation. If fewer normal tonsils are removed now the higher percentage of *Actinomyces* colonies may indicate that they are a sign of disease in the tonsils

Histological examination shows on the other hand no distinct difference between tonsils containing granules and the rest of the material. Fibrosis one of the hallmarks of actinomycosis is not conspicuous. There is therefore not sufficient reason to suppose that chronic tonsillitis is actinomycotic. The possibility should be considered however that the granules may act as foreign bodies like *Soderlund* (1927) claimed they could do in salivary ducts. His theory that salivary duct calculus are calcified *Actinomyces* colonies has on the other hand not been confirmed by *Husted* (1953)

Tonsils as the port of entry in actinomycosis has been described and in a few cases the disease started after tonsillectomy (*Cope* 1938 *Harvey et al* 1959 *Poncet* 1904)

The age difference in the present material compares with the distribution found in actinomycosis. The majority of cases occur between the age of 15 and 35. Less than 3 per cent occur in children under the age of ten (*Cope* 1938)

The sex proportion found 5 males and 12 females does not conform with the distribution in actinomycosis which is something like two males to one female (*Cope* 1938)

SUMMARY AND CONCLUSION

Tonsils from 103 tonsillectomy cases were re examined histologically for *Actinomyces* granules. A positive identification was made in 17 cases. PAS staining was found excellent for screening purposes

The infestation rate was higher after puberty than before and showed an age distribution similar to that in clinical actinomycosis. The sex proportion did not show the male predominance characteristic of the disease

Neither the present nor any other tonsillectomy material is normal. The possibility cannot be excluded that *Actinomyces* colonies may be associated with tonsillar disease either as a cause or as a consequence

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THE SPECIFICITY OF CIRCULATING ANTIBODIES IN EXPERIMENTAL INFECTIONS WITH *MYCOBACTERIUM BOVIS* AND *MYCOBACTERIUM TUBERCULOSIS* DEMONSTRATED BY IMMUNOFLUORESCENCE

By

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In a previous paper (1) the presence of circulating antibodies in rabbits infected with *M avium* and *M bovis* was demonstrated by means of immunofluorescence The antibodies produced during infection with *M avium* possessed a high degree of type specificity which finding led to the elaboration of a method for serological type identification of avian and avian like mycobacteria (2)

The present study concerns investigation into the specificity of antibodies in rabbits infected with *M bovis* (virulent and attenuated (BCG)) and *M tuberculosis* The three parts of which it is composed are

A A methodological study of the fluorescence antibody test (FAT) concluding with a detailed description of the technique used subsequently

B Immunization schedules serum production and specificity of antisera

C Serotyping wild strains of *M bovis* and *M tuberculosis* isolated from Danish patients

PART A

METHODOLOGICAL STUDY OF THE FLUORESCENCE ANTIBODY TEST (FAT)

The technique described in previous papers (1 2) was based mainly on studies with *M avium* and was found inadequate for the investigation of mammalian mycobacteria A positive FAT is read as a yellowish green emission from the bacilli when the smear is excitated with a bluish ultraviolet beam When the FAT is negative *M avium* and most atypical mycobacteria emit a bluish autofluorescence which is easily

distinguishable from the positive reaction while mammalian myco bacteria emit a greyish white autofluorescence which makes differ entiation difficult Furthermore it was found difficult to keep the bacilli on the slides during the different stages of the staining pro cedure This phenomenon was particularly evident in the positive reactions thus resulting in a number of false negatives

Various conditions for the FAT were therefore investigated basing the evaluation of their suitability on the brightness of the bacilli stained by the fluorescent antibodies Records were kept of the background staining and the number of bacilli observed using coded smears

Exact reproducibility was not obtained but the results of parallel investigations always showed the same trends

MATERIALS

Substrate The *M tuberculosis* strain (SSC 891) was used as substrate in all the FAT studies in this section

Antiserum The homologous serum (designated Tub III in Part B) was collected after four consecutive injections the FAT titre was 3160

METHODS AND RESULTS

Table 1 shows the *effect of various fixation methods* using cleaned uncoated slides and slides cleaned and precoated with gelatine (see below), keeping all the other conditions in the test constant

As will be seen from the table the influence of the fixation method did not vary to any extent Both heat fixation and chemical fixation were usable and combinations of heat and chemical fixation offered no advantages However slides precoated with gelatine showed more and generally more brilliant bacilli and a less prominent background than uncoated but cleaned slides On the basis of these results pre coated slides and the most promising of the fixation methods were used in the subsequent experiments

Table 2 shows the *effect of incubation temperature* (22 C and 37 C) in the FAT with antiserum and normal rabbit serum using the five best fixation methods from Table 1 There were no definite differences between the readings at the two temperatures However there was a tendency towards more pronounced background staining with normal rabbit serum in slides incubated at 37 C

In this experiment use of acetone or pyridine fixation seemed ad vantageous and little if any difference between the two methods was seen However as pyridine releases unpleasant and even dangerous vapours that chemical was omitted from subsequent experiments

Table 3 shows the *effect of pH* in the FAT using precoated slides acetone as fixative and an incubation temperature of 22 C It will be seen that a broad pH optimum was found *viz* in the range 7 20 to 7 50 The results with normal rabbit serum show that at higher pH values

TABLE 1

Effect of Fixation Method on the FAT Using Anti M Tuberculosis Serum and Smears from the Homologous Strain

Fixation method	Slides cleaned but not coated with gelatine			Slides cleaned and coated with gelatine		
	Specific staining	Background	No of bacilli	Specific staining	Background	No of bacilli
1 Oven at 80 C overnight	3	+++	+	3-4	++	+++
2 *Oven at 65 C for 2 hours*	4	+++	+	4	++	+++
3 Hot plate at 65 C for 2 hours	4	+++	+	4	++	+++
4 Methanol for 15 minutes	3	++	+	4	+	+++
5 *Ethanol for 15 minutes*		++	+	4	++	+++
6 *Acetone for 15 minutes*	4	++	+	4	++	+++
7 10% formaldehyde for 15 minutes	4	+++	+	3	+++	+++
8 *Pyridine for 15 minutes*	4	++	+	4	++	+++
9 Isopropanol for 15 minutes	3	+++	+	4	++	++
10 5% phenol for 15 minutes	3	+++	+	3-4	+++	++
11 Method 2 + 4	3	++	(+)	3	++	+++
12 Method 2 + 5	4	+++	+	3-4	++	+++
13 Method 2 + 6	4	+++	+	4	++	+++
14 Method 2 + 7	4	++	++	3	+++	++
15 Method 2 + 8	3	++	++	3-4	+++	+++
16 Method 2 + 9	4	++	++	4	++	+++
17 Method 2 + 10	3 4	+++	++	4	+++	+++
18 Method 4 + 2	4	+++	+++	3 4	+++	++
19 Method 5 + 2	3 4	+++	++	4	+++	+++
20 Method 6 + 2	3	+++	+	3-4	+++	+++
21 Method 7 + 2	2-3	+++	(+)	2-3	+++	+++
22 Method 8 + 2	3	+++	+	3-4	+++	+++
23 Method 9 + 2	3 4	+++	++	4	+++	++
24 Method 10 + 2	3	+++	++	3	+++	+++

Fixation methods 1-3 = heat fixation 4-10 — chemical fixation 11-17 = heat fixation followed by various chemical fixation methods 18-24 = chemical fixation followed by heat fixation

Intensity of specific staining Code 0 – 4

Intensity of background staining + – +++

No of bacilli (+) – +++

The fixation methods in italics were used in subsequent experiments

there was a tendency to more intense staining of the bacilli and with both sera there was a more pronounced background colour In subsequent experiments a pH value of 7 35 was used

Additional conditions investigated included the *molarity* (μ = 0 15 was found superior to μ = 0 30 and μ = 0 07) and the *ionic composition of buffers* (0 15 M sodium chloride 0 01 M phosphate was found superior to 0 075 M sodium chloride 0 075 M phosphate and 0 15 M phosphate)

TABLE 2

Effect of Incubation Temperature on the FAT using Normal Rabbit Serum and Anti M Tuberculosis Serum as Middle Layer in Coons's Indirect Technique All Smears Precoated with Gelatine

Fixation method	Normal rabbit serum Specific staining	Back ground	No of bacilli	Antiserum Specific staining	Back ground	No of bacilli
Incubation Temperature 22 C						
2	1	0	+++	4	++	+++
3	1	0	+++	4	++	+++
5	1-2	0	+++	4	+	+++
6	1	0	+++	4§	++	+++
8	1	0	+++	4	++	+++
Incubation Temperature 37 C						
2	1	+	+++	4	++	+++
3	1-2	+	+++	4	++	+++
5	1	0	+++	4	++	+++
6	1-2	+	+++	4§	+	+++
8	1	+	+++	4§	++	+++

Method 2 = oven at 65 C for 2 hours Method 3 = hot plate at 65 C for 2 hours Method 5 = ethanol for 15 minutes Method 6 acetone for 15 minutes Method 8 — pyridine for 15 minutes

§ Bacilli very brilliant

For explanation of symbols see Table 1

TABLE 3

Effect of Increasing pH on the FAT Using Anti M Tuberculosis Serum and Normal Rabbit Serum against M Tuberculosis Smears Precoated Slides Acetone Fixation and Temperature 37 C

pH	Normal rabbit serum Specific staining	Back ground	No of bacilli	Antiserum Specific staining	Back ground	No of bacilli
6.80	1	++	+++	2	+	+++
6.90	2	++	+++	3	+	+++
7.00	1	+++	+++	2	+	+++
7.10	1-2	++	+++	3	+	+++
7.20	1-2	++	+++	4	++	+++
7.30	1-2	++	+++	4	++	+++
7.40	2	++	+++	4	++	+++
7.50	2	+++	+++	4	++	+++
7.60	2	+++	+++		+	+++

For explanation of symbol see Table 1

FINAL FAT TECHNIQUE

On the basis of the results of the methodological study the following technique was used in all subsequent experiments

1 Slides for microscopy (Sovirex®) were rinsed in sulphuric acid dichromate neutralized in ammonium hydroxide rinsed in ion exchanged water and kept in ethanol until use

2 The cleaned slides were coated with 0 5 per cent gelatine air dried fixed in 5 per cent formaldehyde for 10 to 15 minutes rinsed in ion-exchanged water and air dried

3 Colonies from tubes with Lowenstein Jensen medium were emulsified in buffered saline (BS = 0 15 M saline 0 01 M phosphate pH 7 35) A drop of the suspension was placed on the coated slide which was air dried before fixation in acetone for 15 minutes at room temperature (about 2? C) The acetone was evaporated from the slides under a fan the process being continued for at least 30 minutes after the disappearance of any visible acetone

Indirect Technique

4 A drop of antiserum was placed on the smear and distributed evenly with a wooden pin
5 The slides were incubated in moist chamber for 30 minutes at room temperature
6 The slides were rinsed in BS for 10 minutes and the surplus fluid removed from around the smear by means of a paper tissue
7 A drop of diluted goat anti rabbit globulin conjugated with FITC was placed on the smear and distributed
8 Incubation as mentioned under 5
9 Rinsing as mentioned under 6
10 The slides were mounted with ether rinsed coverslips in glycerol buffered at pH 9

Direct Technique

A drop of antiserum conjugate was placed on the smear and distributed as mentioned under 4 above The subsequent stages were as in points 5 6 and 10 above

Conjugation of antisera with FITC was performed according to *Cherry et al* (5)

Readings were made as described previously (1) and the results recorded as Codes 0 to 4 (*) Only results designated as Codes 3 and 4 were regarded as positive All readings were performed blindly with coded slides

PART B

IMMUNIZATION SCHEDULES SERUM PRODUCTION AND SPECIFICITY OF ANTISERA

The infection of rabbits with *M bovis* *M tuberculosis* and BCG causes three different immunological situations With *M bovis* a virulent infection can be provoked and on the basis of previous experience we used anti *M bovis* sera collected early in the infection period (1) A more prolonged course was necessary for the production of anti *M tuberculosis* and especially anti BCG sera

MATERIALS

Bacterial strains for serum production The strain of *M bovis* (E 4584/64) was the same as that used in previous investigations (1) The *M tuberculosis* strains (SSC 8 1 SSC 856 SSC 899) were selected on the basis of preliminary results which showed serological difference between them

M bovis var BCG = ATCC 19?74

Bacterial strains for specificity determination The 13 strains used form part of Series 1 issued by the International Working Group on Mycobacterial Taxonomy (see Table 5 below)

TABLE 4

Immunization Schedule for Production of Antisera

Designation of sera	Strain used for immunization	No of injections	Time of injections (days after onset)	Inoculum	Time of bleeding (days)	Titres of sera	Titres of final pools of sera
Anti M *bovis* I	D 4584/64	1	0	1 ml iv	14	100 – 316	316 †
Anti M *bovis* II	D 4584/64	8	0 3 7 10 14 17 21 24	1–2 ml §	28	< 10	< 10
Anti BCG	ATCC 19274	4	0 7 14 21	0 1–1 ml	28	316	316
Anti M *tuberculosis* I	SSC 821	1	0	1 ml iv	28	100 – 1000	316
Anti M *tuberculosis* II	SSC 856	3	0 7 14	0 1 ml iv 0 1 ml iv 1 ml sc	39	100 – 316	316
Anti M *tuberculosis* III	SSC 891	4	0 7 14 21	0 1–1 ml	28	1000 – 3160	3160 †
Anti M *tuberculosis* IV	SSC 829	3	0 64 129	0 1 ml iv	180	316 – 1000	1000

Sera used in the indirect technique   † Sera used in the direct technique   § killed bacilli

METHODS AND RESULTS

Immunization and production of serum The strains were grown in Dubos liquid medium with Tween 80 *Before inoculation* the cultures were adjusted to an optical density of 0 1 in Coleman Junior spectrophotometer at 595 nm using 20 mm cuvettes Given volumes of these suspensions were inoculated into groups of four rabbits according to the immunization schedule shown in Table 4 During the immunization *period* blood samples were drawn at intervals from the ear vein and the FA titres against homologous strains were estimated At the end of the immunization periods the animals were bled by heart puncture and the serum collected Phenol and EDTA were added to final concentrations of 0.5 per cent and merthiolate to 0 01 per cent The sera were stored at +4 C or —20 C.

Anti M bovis Sera

Anti M bovis I serum (Bov I) was produced by a single injection of viable bacilli Among the four sera obtained two showed a FA titre of 316 and two a titre of 100 The first named sera were pooled and the FA titre of the pool was 316

Anti M bovis II serum was collected following hyperimmunization with phenol killed bacilli None of the sera gave positive FA reactions

The *BCG* immunization consisted of four consecutive injections with viable bacilli Among the four rabbits used two died following the third injection and one following the fourth The surviving rabbit was bled and the FA titre of its serum was 316

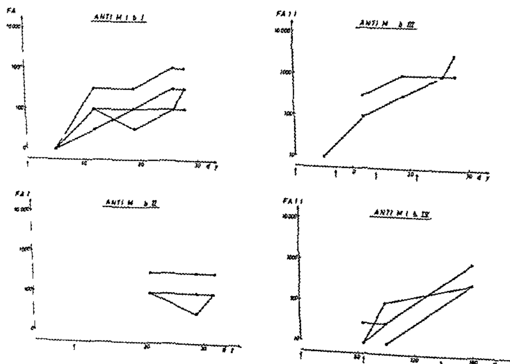


Fig 1

Titre curves of sera from rabbits injected with *M tuberculosis* according to immunization schedule shown in Table 4 Arrows indicate time of injection Titre is defined as the reciprocal of the last dilution still giving positive reading in the indirect FAT

TABLE 5

Comparison of Specificity of Different Sera in the Indirect FAT

Substrate		Serum					
Designation	Strain No	Bov I	BCG	Tub I	Tub II	Tub III	Tub IV
Strains used for serum production							
M bovis	I 4584/64	+	+	—	—	+	+
M bovis (BCG)	ATCC 19274	+	+	+	+	+	+
M tuberculosis	SSC 821	+	+	+	+	+	+
M tuberculosis	SSC 856	+	+	+	+	+	+
M tuberculosis	SSC 829	+	+	+	+	—	+
Series issued by International Working Group on Mycobacterial Taxonomy							
M avium	ATCC 15769	—	—	—	—	+	—
M intracellularis	ATCC 13950	—	—	—		—	—
M kansasii	ATCC 12478		—	—	—	—	—
M marianum	ATCC 19275	—		—	—	—	—
M bovis	ATCC 19210	—	—	—	—	+	—
M bovis (BCG)	ATCC 19274	+	+	+	+	+	+
M marinum	ATCC 927	—		—	—	—	—
M fortuitum	ATCC 6841	—	—	—	—	—	+
M smegmatis	ATCC 14468	—		—	—	—	+
M phlei	ATCC 19249			—	—	—	—
M vaccae	ATCC 15483			—	—	—	—
M xenopei	ATCC 19970			—	—	—	—
M microti	ATCC 19422	—		—	—	—	—

Anti M tuberculosis Sera

Four different methods were employed for the production of anti *M tuberculosis* sera using viable bacilli. Titre curves during immunization are shown in Fig 1.

Anti M tuberculosis serum (Tub I) was produced by a single intravenous injection. The four sera had FA titres of 1000, 316, 316 and 100 respectively. The first two of these were pooled and the resulting titre was 316.

Anti M tuberculosis II serum (Tub II) was produced by two consecutive intravenous injections followed by one subcutaneous injection. Among the four sera two had a FA titre of 316 and two a titre of 100. The first two sera were pooled and the resulting titre was 316.

Anti M tuberculosis III serum (Tub III) was produced by four consecutive injections. Among the four rabbits used two died following the fourth injection. The two surviving rabbits had FA titres of 3160 and 1000 and the titre of the pooled sera was 3160.

Anti M tuberculosis IV serum (Tub IV) was produced by three consecutive intravenous injections of small doses at long intervals. Among the four sera one had a FA titre of 1000 and three a titre of 316 after 180 days. The first named serum was used in the specificity comparisons.

Specificity Determination of Sera

Table 5 shows the results of the FAT using the different mycobacterial strains and the six sera diluted to the penultimate titration stage.

In this experiment the sera designated Tub I and Tub II reacted with BCG and *M tuberculosis* exclusively. Serum Tub III reacted also with *M avium* and *M bovis*. Serum Tub IV reacted with *M fortuitum* and *M smegmatis*. When all the sera were tested repeatedly against other *M fortuitum* strains they all gave positive reactions with a frequency of about 10 per cent.

These results were decisive for the selection of the two anti *M tuberculosis* sera (Tub I and Tub II) for use in the indirect technique in Part C.

PART C

SEROTYPING WILD STRAINS OF *M. BOVIS* AND *M. TUBERCULOSIS*

For the practical application of the FAT in the laboratory diagnosis of tuberculosis it is essential to have a knowledge of the serological relationship between mammalian mycobacteria. Preliminary results showed some differences between *M. bovis* and *M. tuberculosis* and to a certain degree also within the latter species. A number of wild strains isolated from Danish patients with tuberculosis were therefore tested against some of the sera mentioned in Part B.

MATERIALS

Bacterial strains tested by the sera (substrate). 104 strains of *M. tuberculosis* and 42 strains of *M. bovis* were selected from strains isolated at the Tuberculosis Department of Statens Seruminstitut, Copenhagen, in 1965. Before testing they were kept in tubes with Löwenstein-Jensen medium at about [illegible] C for one to four months. Smears were made direct from these stock cultures.

Antisera. The sera designated Bov I, BCG, Tub I and Tub II in Part B were used in the indirect technique and conjugates of Bov I and Tub III in the direct technique.

METHODS

For details of the methods used, see Part A.

RESULTS

Tables 6 and 7 show the results of serotyping 146 strains (104 *M. tuberculosis* and 42 *M. bovis*) using the indirect fluorescence technique.

Table 6a shows the distribution of the results when Tub I and Tub II were used as test sera. Among the 17 inconsistent results with the *M. tuberculosis* strains, four were read as — Tub I + Tub II. This distribution has a p value of 4.9 per cent in a binomial distribution.

TABLE 6

Distribution of Results when 104 M. tuberculosis *and 42* M. bovis *Strains Tested against* M. tuberculosis *Antisera (a) and* M. bovis *and BCG Antisera (b) in the Indirect FAT*

	Antisera	Strains	
		M. tuberculosis	*M. bovis*
(a)	— Tub I — Tub II		30
	— Tub I + Tub II	4	4
	+ Tub I — Tub II	13	5
	+ Tub I + Tub II	87	3
(b)	— Bov — BCG	3	3
	— Bov + BCG	8	4
	+ Bov — BCG	8	4
	+ Bov + BCG	85	31

with $n = 17$ and $p = 0$. (The statistical evaluation of such distributions has been described previously (3)) The negative frequencies with the two sera are about 9 and 17 per cent respectively In the *M bovis* material, about 80 per cent of the strains did not react with the sera

Table 6b shows the corresponding results when Bov I and BCG were used as test sera The two sera reacted equally well with negative frequencies of 10 and 16 per cent in the *M tuberculosis* and *M bovis* material respectively

Table 7a shows the distributions when the reactions with the two anti *M tuberculosis* sera are regarded as one double reaction and where only one positive reading permits notification of a result as positive Correspondingly the reactions with Bov I and BCG are regarded as one double reaction In the *M tuberculosis* material anti *M tuberculosis* serum and anti *M bovis* serum reacted equally well However in the *M bovis* material the superiority of anti *M bovis* serum as regards staining efficiency is significant

TABLE 7

Evaluation of Results from Table 6

	Antisera	Strains	
		M tuberculosis	*M bovis*
(a)	— Tub — Bov	1	3
	— Tub + Bov	4	27
	+ Tub — Bov	2	0
	+ Tub + Bov	97	12
(b)	— Tub — Bov	7	10
	— Tub + Bov	15	29
	+ Tub — Bov	12	1
	+ Tub + Bov	70	2

Two reactions with *M tuberculosis* antisera are regarded as one test and two reactions with *M bovis* antisera as one test One positive result permits positive notification in (a) and both results must be positive for positive notification in (b)

Table 7b shows the corresponding results with the double reactions when positive readings from both reactions are necessary for a positive notification

Tables 8 and 9 show the results of testing the 42 *M bovis* strains by the direct immunofluorescence technique

Table 8a shows the distribution of results when the strains are tested twice against the anti *M tuberculosis* conjugate The average negative frequency of the reactions is 67 per cent and the distribution of inconsistent results (n = 6 and 10) may be fortuitous Table 8b shows the corresponding results when the strains are tested twice against anti *M bovis* conjugate The negative frequency is about 12 per cent for each reaction

Table 9a shows the results when the two consecutive reactions with the anti *M tuberculosis* conjugate and the anti *M bovis* conjugate are regarded as double reactions and where one positive reading per double reaction permits notification of a result as positive In Table 9b both readings in the double reaction must be positive for a positive notification In both cases the results demonstrate the superiority of *M bovis* conjugate for staining *M bovis*

Finally mention should be made of a preliminary series of investigations where 485 strains of *M tuberculosis* were tested once against the *M tuberculosis* conjugate The negative frequency in that series was 9 per cent

TABLE 8

Distribution of Results when 42 M bovis *Strains Tested Twice against Anti* M tuberculosis *Conjugate (a) and anti* M bovis *Conjugate (b) in the direct FAT*

	Conjugate	*M bovis* strains
(a)	— tub 1 — tub 2	18
	— tub 1 + tub 2	6
	+ tub 1 — tub 2	10
	+ tub 1 + tub 2	8
(b)	— bov 1 — bov 2	0
	— bov 1 + bov 2	5
	+ bov 1 — bov 2	5
	+ bov 1 + bov 2	32

TABLE 9

Evaluation of Results from Table 8

	Conjugate	42 *M bovis* strains
(a)	— tub — bov	0
	— tub + bov	18
	+ tub — bov	0
	+ tub + bov	24
(b)	— tub — bov	9
	— tub + bov	25
	+ tub — bov	1
	+ tub + bov	7

Two reactions with anti *M tuberculosis* conjugate are regarded as one test and two reactions with anti *M bovis* conjugate as one test One positive result permits positive notification in (a) and both results must be positive for positive notification in (b)

DISCUSSION

The aim of this study was to examine the specificity of circulating antibodies produced by infection with viable tubercle bacilli Since rabbits were used for antibody production a virulent infection due to

M bovis could be provoked and antibodies obtained early in the infection could be examined on the lines of previous investigations (1 2) A corresponding production of antisera against *M tuberculosis* and BCG was not thought to be possible and different immunization schedules were therefore adopted in order to feign the multiplication of bacilli in the virulent infection It was possible by repeated injections of viable bacilli to produce antibodies against *M tuberculosis* and BCG in rabbits The technical problems involved the risk of death of the animal within one to two days after the later injections and the possibility of obtaining an antibody response against minor antigens due to the prolonged immunization schedule One of the sera used was produced by a single injection of viable bacilli and showed a high degree of type specificity though a long immunization period was necessary

The specificity of the antibodies obtained against different mycobacterial strains was reasonably good as will be seen from Table 5 Anti *M tuberculosis* III gave reactions with the *M avium* type strain and consequently was not used in the indirect technique However on account of its high titre this serum was used in the direct technique The other sera employed gave no cross reactions with such important bacteria as *M avium*, *M intracellularis* *M kansasii* and *M xenopei*, but all of them reacted occasionally with *M fortuitum* Thus other types of sera might be necessary for use in routine laboratory typing

The fluorescence antibody technique used was based on the results of a methodological study performed in an attempt to decrease the number of false negative results An important detail of the technique is the use of precoated slides which during the subsequent manipulations retain the fixed bacilli better than cleaned but uncoated slides Pyridine and acetone gave the best results but because of the unpleasant vapours liberated by pyridine acetone was preferred The final technique used in the subsequent typing still gave a certain number of false negatives which necessitated a statistical evaluation of the results In routine typing the examination of a number of parallel slides would be required

In serotyping the *M tuberculosis* and *M bovis* strains the frequency of negative results and the distribution of the inconsistent results were decisive for the evaluation All the sera examined showed the same trend as regards staining property Anti *M bovis* sera (including BCG) stained *M tuberculosis* and *M bovis* while anti *M tuberculosis* sera generally stained *M tuberculosis* only

The opinions of various workers differ concerning serological differentiation between mammalian mycobacteria Most workers do not distinguish between *M tuberculosis* and *M bovis* but a few reports present possibilities for differentiation similar to those found in this study Schaefer (6) working with polysaccharide and protein antigens from culture filtrates found a protein antigen in the filtrate from bovine

strains that was not present in filtrates from BCG or human strains Later he reported the same possibility of differentiation with a hydrochloric acid extract from whole bacilli (7) By means of immuno electrophoresis and immunodiffusion *Castelnuovo et al* (4) revealed difference between *M tuberculosis* and *M bovis* The latter possessed an antigen that was not found in the former or in BCG Furthermore another antigen found both in *M tuberculosis* and *M bovis* was lacking in BCG

The difference found in the present study seems to be parallel to that described by *Schaefer* and might be explained in the same way viz the existence of an antigen common to *M bovis* and *M tuberculosis* and an antigen specific for *M bovis* However we found that BCG was able to induce antibody production against the specific *M bovis* antigen

For the practical application of these results it is necessary to place *M bovis* in one serotype and *M tuberculosis* in another so far as immunofluorescence work is concerned In the material examined we found no definite evidence of serological variations within the two species The different strains of *M tuberculosis* employed in serum production were selected on the basis of preliminary results where variations were found As mentioned in Part C the difference between the two anti *M tuberculosis* sera was on the borderline of statistical significance and thus the serological variation if any must be slight The Bov I and BCG sera showed a slightly higher frequency of negative results with *M bovis* than with *M tuberculosis* This phenomenon might be explained by a higher degree of variation in the *M bovis* strains However the conditions for the FAT were established in experiments with *M tuberculosis* and those conditions might be less favourable for *M bovis* strains

If the immunofluorescence technique is to be employed in routine laboratories for the specific staining of tubercle bacilli anti *M bovis* serum should be used in order to stain both *M tuberculosis* and *M bovis* and anti *M tuberculosis* sera stained strains of *M tuberculosis* with equal efficacy but that anti *M bovis* serum was significantly more suspected tuberculosis patients smears from *M tuberculosis* should be used as substrate or alternatively smears from both species must be employed

SUMMARY

Circulating antibodies in rabbits infected with *M bovis* and *M tuberculosis* were examined from the point of view of their specificity in the fluorescence antibody test (FAT) Statistical evaluation of the results of serotyping strains from tuberculous patients showed that anti *M bovis* and anti *M tuberculosis* sera stained strains of *M tuberculosis* with equal efficacy but that anti *M bovis* serum was significantly more effective in staining *M bovis* strains

The conditions for the IAT were examined in a methodological study the results of which are included in this paper

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STUDIES ON THE QUANTITATION OF LYMPHOCYTE RESPONSE *IN VITRO*

By

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Received 11 xii 68

Submitted to suitable culture conditions blood lymphocytes can be induced to proliferate by several stimuli such as unspecific mitogens antigens against which the lymphocyte donor is sensitized antibodies against determinants on the lymphocyte surface and transplantation antigens both in the form of intact cells and as cell free preparations

The present investigation is based on a method of culture which allows good cell survival and early occurrence of significant thymidine incorporation in the stimulated cultures Three kinds of stimulation have been used 1) phytohaemagglutinin (PHA) as an example of an unspecific mitogen 2) purified tuberculin and 3) mixed cultures of allogeneic cells

Several investigators (2 3 5 9 11) have stressed the importance of standardizing culture conditions and assay methods Lack of reproducibility has been a major problem in quantitation of the lymphocyte response by means of morphological methods such as the counting of transformed cells and mitoses or of labelled cells in autoradiographs Therefore a systematic study of the rate of thymidine incorporation during 7 8 days of culture was undertaken A rapid and accurate quantitation was obtained by means of a modification of the assay method proposed by *Mosedale & Parke* (8)

It has been shown that the maximal thymidine uptake in cultures of the same type may occur on different days Hence determination of the thymidine incorporation at a fixed time when the peak response is supposed to occur may be misleading Fortunately however it appears that the response is best quantitated by measurements during the first days of proliferation i e before the peak response occurs

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MATERIAL AND METHODS

Preparation of the Cultures

50 ml of blood from normal adults is defibrinated for 10 minutes in an Erlenmeyer flask containing 10 to glass beads and mixed with equal volumes of the following solution 7 parts of TC 199 (Glaxo) containing penicillin and streptomycin and 3 parts of dextran 6 per cent in 0 9 per cent sodium chloride (Intradex Glaxo) After sedimentation for 30 60 minutes at 37 C 50 60 ml of supernate can be removed

The sediment is centrifuged (1000 g 15 minutes) for preparation of cell free medium

The number of lymphocytes and granulocytes in the leucocyte suspension is determined by phase contrast microscopy using an ordinary haem cytometer The concentration of lymphocytes is adjusted to 1 million per ml either by concentration of the suspension by centrifugation (200 g 10 minutes) or by dilution with cell free medium Normally it is possible with this method to obtain 40–80 million lymphocytes with a granulocyte content of 0 5–2 million per million lymphocytes

The suspension is transferred to culture vials usually 2 or 5 ml per vial Brown screw capped medicine bottles have been used delivered a eptic from Pl3t Manufaktur Limmared Sweden The volume of the vial is 10 ml the internal diameter being 19 mm

The gas pha e of the vials is 5 per cent CO in atmospheric air The cultures are maintained at 37 C

Stimulation of the Cultures

Reconstituted PHA (Burroughs Wellcome) or purified tuberculin 0 02 mg per ml (Statens Seruminstitut Copenhagen) is added to the cultures in the concentrations stated Stimulation with tuberculin was investigated in cultures from person with a positive tuberculin skin test

Mitogen is added to the remaining cell free medium in the same concentration as in the cultures and it is stored at 4 C for later change of medium

Mixed cultures are prepared using equal volumes of leucocyte suspension from two unrelated persons total volume 2 or 5 ml per culture

Change of Medium

Unless otherwise stated change of medium is performed on days 2 4 and 6 The upper half of the culture medium is removed without disturbing the cells at the bottom of the vial and is replaced by an equal volume of cell free medium pre warmed to 37 C The cultures are re flushed with 5 per cent CO in air before the caps are closed

Harvest of the Cultures

The cultures are terminated at 24 hour intervals After a careful resuspension of the cells 1500 μl is transferred to new culture vials containing radioactive tracer

The remaining suspension is used for viability testing and smears

After incubation with tracer for 240 minutes at 37 C the cells are resuspended and 1000 μl is collected on Whatman glass fibre filters type GF/C, diameter 12 8 mm mounted in Millipore filterholders connected to a water suction system

The rest of the labelled suspension is used for autoradiography

The cell deposit on the filter is treated successively with 60 ml of isotonic saline 40 ml of ice cold trichloroacetic acid and 40 ml of methanol

In preliminary experiments the filters were counted directly with the cell coated surface upwards in 15 ml of dioxan scintillator (naphtalene 60 g methanol 100 ml ethylene glycol 20 ml PPO 5 g dimethyl POPOP 0 3 g made up to 1 litre with dioxan)

In all subsequent experiments the active cell material on the filters has been digested with hyamine (10) The dried filters are transferred to counting vials and 1000 μl of hyamine (Packard) is added After 24 hours in the dark at 37 C 15 ml of toluene scintillator (PPO 5 g + dimethyl POPOP 0 3 g per litre toluene) is added The samples are counted in a Packard Tri Carb Liquid Scintillation Counter

Type 314 EX 1 to a counting error below 1 per cent All results are expressed as counts per minute per 1 million lymphocyte of the initial culture The background counts are subtracted from all data

Tracer

Thymidine 2 ^{14}C (specific activity 60 5 mCi/mM) 0 1 µCi per ml culture, Uridine 2 ^{14}C (specific activity 58 1 mCi/mM) 0 1 µCi per ml culture and DL leucine 2 ^{14}C (specific activity 51 mCi/mM) 1 0 µCi per ml of culture have been used All tracers have been purchased from The Radiochemical Centre Amersham England

Viability Counting

Viability counting is performed by direct counting of living and dead cells under phase contrast illumination in an ordinary haemocytometer The dead cells are characterized by cytoplasmic oedema and nuclear pyknosis No results of the viability counts during the first 3 days are given as the presence of polymorphs disturbs the counting during this period

RESULTS

Assay Method

When assaying the incorporation of ^{14}C labelled tracers the cells can be deposited on a glass fibre filter and counted directly in a dioxan scintillator (8) With this method the activity remains localized to the filters (10) It is however possible to dissolve the active cell material on the filters with hyamine In the case of non hyamine treated filters the highest counting efficiency was obtained using dioxan scintillator while hyamine treated samples were counted with the greatest efficiency using toluene scintillator

It is seen (Table 1) that the hyamine treated filters—*in spite of* hyamine quenching—constantly gave higher counts than the non hyamine treated filters After turning the non hyamine treated filters with the cell side downwards the count rate decreased by 5–10 per cent When the non hyamine treated filters were transferred to new counting vials the activity was transferred with the filters while the activity remained in the counting vials after removal of the hyamine treated filters showing that the active cell material was dissolved

Both methods showed a linear relationship between the amount of labelled cells collected on the filters and the activity found and both methods may therefore be used The hyamine method which was worked out primarily for counting of ^{3}H labelled tracers (10) was preferred for counting of ^{14}C labelled tracers too due to the higher counting efficiency and the independence of geometrical factors

Change of Medium

The survival and proliferation of the cells are improved considerably by change of medium Fig 1 shows the thymidine incorporation and number of living cells in mixed cultures when in one set of vials change of medium had been performed on days 2 4 and 6 while in the control set only flushing of the cultures with 5 per cent CO_2 in air was

TABLE 1
Comparison between Assays of Non hyamin Treated and Hyamin Treated Filters

Culture No	µl on filter	Assay method	c p m	c p m after turning the filter with cells down wards	% fall	c p m after removal of filter	% fall	c p m in new vials
3/71	500	H	3077			2983	1 5	1,3
	500	U	2924	2662	9	1		2631
	1000	H	6274			61/6	1 5	60
	1000	U	5700	5319	6 5	3		5913
	1500	H	9447			9391	0 7	343
	1500	U	8438	7746	8	4		8537
	2000	H	12927			12786	1 1	383
	2000	U	11495	10761	6 5	2		10533
3712	500	H	4366			4382	0	163
	500	U	4213	3800	10	24		3845
	1000	H	8961			9006	0	137
	1000	U	8271	7780	6	122		7655
	1500	H	13459			13193	2 0	176
	1500	U	12012	11430	5	40		11146
	2000	H	18262			18176	0 5	333
	2000	U	17010	15330	10	9		15461

H Hyamin treated filters counted in 15 ml of toluen scintillator
U Non hyamin treated filters counted in 15 ml of dioxan scintillator

Two different pools of PHA stimulated cultures were harvested on filters in the amounts indicated 24 hours after addition of scintillation fluid the non hyamin treated filters were turned with the cell side downwards 48 hours after the addition of scintillation fluid all filters were removed from the original counting vials and transferred to new vials (the orientation of the filters in the new glasses was not controlled)

done on the same days It is seen that due to a higher proliferative activity twice as many living cells were found in the cultures on day 8 when the medium was changed as compared with control cultures Surprisingly daily change of medium did not further improve neither cell survival nor thymidine incorporation *Change of medium on day 3* only gave less than optimal activity These results held true in both PHA and tuberculin stimulated cultures

The culture medium was diluted by 30 per cent Intradex To investigate whether the need for change of medium was due to the decreased concentration of nutritive factors in the media PHA and mixed culture experiments were performed in which the cells were isolated by centrifugation and cultured in medium with and without *Intradex* No significant difference was observed implying that culturing of cells in undiluted medium could not substitute for change of medium

The addition of 2 5 or 5 mg of glucose to the cultures instead of change of medium on days 2 4 and 6 did not increase the activity above that obtained in cultures in which the medium was not changed

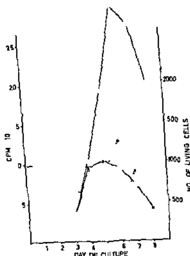


Fig 1

A comparison between mixed cultures with and without change of medium
O Change of medium
X No change of medium.
Dotted lines Number of living cells
Fully drawn lines Thymidine incorporation

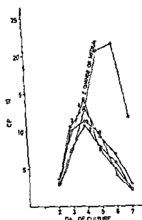


Fig 2

Mixed cultures Increasing volumes of medium have been added to different cultures while the medium has been changed in one culture
See text. The numbers indicate the volume of culture in ml

In stimulated cultures the pH of the medium decreases To investigate whether the effect of the change of medium was due to a partial normalization of the pH in the cultures triplicate mixed cultures were set up To one series bicarbonate was added on days 2 4 and 6 restoring neutral pH as estimated by the colour of the indicator This resulted in a thymidine incorporation higher than that in the control cultures but the activity did not reach the same level as in the cultures in which change of medium was performed

To investigate the possibility that the medium might be exhausted due to an excessive concentration of cells the following experiment was made Equal volumes of leucocyte suspensions from two donors were mixed and dispensed into 5 groups of 6 culture vials each containing 2 ml To the 2nd 3rd and 4th group was added 1 2 and 3 ml of cell free medium respectively so that the final lymphocyte concentration in the 5 groups were 1 00 0 67 0 50 0 40 and 1 00 millions per ml Change of medium was made in the cultures of the 5th group only while the remaining cultures were flushed with 5 per cent CO_2 in air on the same days It is seen (Fig 2) that no significant differences were found in the cultures in which no change of medium was performed irrespective of cell concentration while the cultures in which the medium was changed showed much higher and longer lasting thymidine incorporation

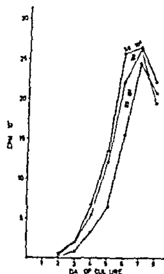


Fig 3

A comparison between mixed cultures with different numbers of cells per culture See text

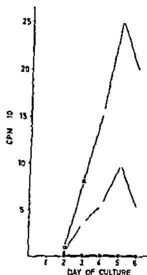


Fig 4

Cultures from a tuberculin hypersensitive person which have been stimulated with different concentrations of purified tuberculin

X–X 0 04 µg per ml
□–□ 0 2 µg per ml
■ ■ 1 µg per ml

The results in Fig 2 also imply that the height of the medium in the cultures in which no change of medium has been performed was of no importance for the thymidine incorporation The same finding has consistently been made in cultures in which the medium was changed

Number of Cells per Culture

To evaluate the significance of the cell density at the bottom of the culture vials mixed culture experiments were made in which the cell concentration was kept constant *i e* 0 5 million per ml while the number of lymphocytes per culture were 0 5 2 and 3 million respectively (Fig 3) It is seen that no significant difference in thymidine incorporation between cultures containing 2 and 3 million lymphocytes was found while the thymidine incorporation in vials containing 0 5 million lymphocytes was delayed compared to the cultures with higher cell density

In other mixed culture experiments and in experiments with tuberculin stimulation in which the lymphocyte concentration was 1 million per ml no difference in the thymidine incorporation was found in vials containing 2 and 5 million cells

On the basis of these experiments it is not possible to determine the optimal density of cells per square unit (7) as the bottom of the vials

is slightly curved so that the cells settle with the highest density along the periphery

Concentration of PHA and Purified Tuberculin

The optimal concentration of PHA was found to be 0 015 ml per ml of culture

The optimal concentration of purified tuberculin varied from individual to individual and must be determined in each case Fig 4 shows a tuberculin titration In other experiments a similar small difference between the optimal concentration and the toxic concentration was found The optimal concentration varied between 0 2 and 1 5 μg of purified tuberculin per ml of culture

Protein RNA and DNA Synthesis

In PHA stimulated cultures significant RNA and protein synthesis is seen within one hour after the initiation of culture whereas DNA synthesis commences at 24 hours (6) The time course of RNA protein and DNA synthesis in antigen stimulated cultures is shown in Figs 5a and 5b The observed parallellism between the incorporation of uridine leucine and thymidine was also found in cultures with weaker responses On the first day of culture significant incorporation has never been encountered

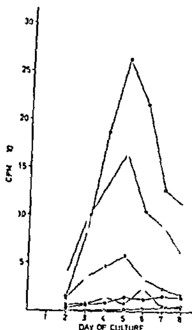
In most of the figures thymidine incorporation by the unstimulated control cultures has been omitted The results depicted in Figs 5 and 7 are however representative It is seen that the activity in the control cultures was slightly increasing towards the 8th day As a rule the thymidine incorporation in the controls was about 200–500 CPM In a few control cultures an increase to 1 000 CPM was seen on days 7 and 8 As seen from Fig 5 a considerable incorporation of leucine and uridine was found in unstimulated cultures

These results indicate that assay of DNA synthesis is the most suitable parameter for quantitation of the lymphocyte response

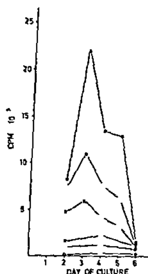
Reproducibility

In order to assess the *variation between individual vials* in mixed cultures and tuberculin stimulated cultures the coefficient of variation was calculated on the basis of 44 determinations in duplicate and 14 determinations in triplicate The result was 10 2 per cent for mixed cultures and 9 6 per cent for tuberculin stimulated cultures which gave a weighted mean of 10 0 per cent

Four out of these 58 experiments showed clearly deviating results (coefficients of variation 21 5 25 4 26 1 and 32 4 per cent) suggesting some unknown technical error If these four experiments were disregarded the coefficient of variation was reduced to 7 0 per cent 7 6 per cent and 7 2 per cent respectively



5a



5b

Fig 5

^{14}C Leucine ^{14}C Uridine and ^{14}C Thymidine uptake in leucocyte cultures
X–X ^{14}C Leucine O–O ^{14}C Thymidine ● ● ^{14}C Uridine
5a Mixed cultures 5b Cultures from a strongly tuberculin hypersensitive person stimulated by 0 2 μg purified tuberculin per ml
Three lower lines show the average activity of the unstimulated cultures

The *filter to filter variation* was estimated on the basis of 49 experiments in duplicate The coefficient of variation was 3 3 per cent

To get an impression of the *day to day variation* mixed cultures from the same two persons were prepared at intervals of 3 days The result is seen from Fig 6

Cell Death—Peak Response

A leucocyte culture is both a growing and a dying culture Among the granulocytes the polymorphs die during the first days but the eosinophils survive until late in the culture The number of living lymphoid cells decreases from the first day of culture at a rate which is largely determined by the culture conditions (Fig 1) When stimulation causes intensive proliferation a transitory increase in the number of living cells occurs see Fig 7 After day 8 a rapid decrease in the number of living cells always occurs in the culture system described Figs 1 and 7 show furthermore that the thymidine incorporation by the stimulated cultures begins to decrease at a time when *the number of living cells* in the cultures is still increasing showing that thymidine incorporation is inhibited already before massive cell death occurs

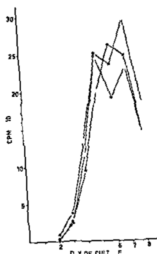


Fig 6

Mixed cultures prepared from the same two test persons with an interval of 3 days
The points represent the average of duplicate or triplicate determinations

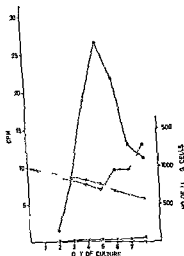


Fig 7

Thymidine incorporation and number of living cells in mixed cultures and controls
Fully drawn lines Thymidine incorporation
Dotted lines Number of living cells
O–O Mixed cultures
X–X Average of unstimulated controls

Fig 8 shows the thymidine incorporation in the experiment described in Fig 5b compared with the thymidine incorporation in cultures from a person who showed a weaker skin test reaction to purified tuberculin It is seen that the stronger skin reactor showed an early and violent response with a peak already on the 3rd day of culture while the weaker reactor showed a slower but still increasing thymidine incorporation on day 5 In other cultures stimulated by tuberculin, the peak response was found on day 4 or 5

In PHA stimulated cultures peak responses were found on day 2 or 3

In mixed cultures in which change of medium was performed (cf Figs 1 2 3 and 6) the peak response was found from day 5 to 7

DISCUSSION

When incorporation of DNA precursors is employed to quantitate the proliferative response of lymphocytes *in vitro,* the measurement is often carried out at a fixed time when the response is believed to be maximal *e g* on the 3rd or 4th day in PHA stimulated cultures on the 5th or 6th day in tuberculin stimulated cultures and on the 6th or 7th day in mixed cultures The present investigation shows however that the

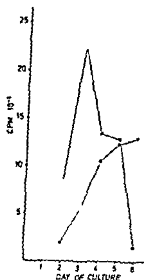


Fig 8

Thymidine incorporation in cultures from two different test persons stimulated by purified tuberculin

○-○ The same person as in Fig 5b who by skin test using 0 02 μg purified tuberculin showed reddening and edema of the whole antebrachium

●-● A person who by skin test using 0 02 μg purified tuberculin showed reddening and induration in an area of 1 × 1 cm

Both cultures were stimulated by 0 2 μg purified tuberculin which in other experiments was found to be the optimal concentration for both test persons.

peak response may occur on different days when the same antigenic stimulant is applied to lymphocyte cultures from different persons

Ilves (1) has recently reported improved cell survival in mixed cultures from inbred strains of rats by change of medium in the cultures In cultures of human lymphocytes too change of medium will improve cell survival and thymidine incorporation (Figs 1 and 2) The characteristic finding is that the peak response is thereby increased and it occurs later in the culture period thus demonstrating that peak response is determined not only by the proliferation of the cells but is also greatly influenced by culture conditions

Furthermore Figs 1 and 2 demonstrate that the thymidine incorporation on day 3 and 4 is identical in cultures from the same individuals whether or not change of medium has been performed suggesting that the early response is much less influenced by the culture conditions and therefore a better measure of the cellular response

In mixed cultures from inbred strains of rats and their F_1 hybrids *Wilson et al* (12) have shown that new cells are continuously entering mitosis for the first time from day 2 to day 7 and that the cells in mitosis then proceed through a successive series of divisions with a constant generation time In mixed cultures from man *Wilson et al* have found a generation time of 14 hours

The immunological significance of the finding that some cells do not start proliferation until late in the cultures has not yet been clarified However due to the short generation time the response will be dominated by the cells which begin proliferation early while cells which enter into mitosis late in culture contribute only to a small extent to the total thymidine incorporation

That the exponential rise of the thymidine incorporation curve levels off before the peak is reached shows that some of the cells already at this time fall out of the mitotic cycle The reason for this is presumably cell death which at least in part is due to suboptimal culture conditions

In the quantitation of the lymphocyte response it therefore seems more reasonable to measure the thymidine incorporation during the first days of proliferation rather than the peak response Fig 4 shows that the optimal tuberculin concentration can be determined on days 3 and 4 as well as later in the cultures Fig 8 shows that a person with a violent reaction to tuberculin skin test shows an early strong response *in vitro* in comparison to a person with a smaller degree of sensitivity and that assessment on day 5 only would have been misleading

In mixed culture experiments the response can likewise be quantitated during the first three to four days of culture Investigations are at present in progress using one way cultures between HL-A typed family members in order to determine the correlation between the early response *in vitro* and the degree of HL-A incompatibility

SUMMARY

The present investigation was undertaken in an attempt to define the optimum conditions for quantitation of the lymphocyte response *in vitro*

The cellular uptake of ^{14}C-thymidine was measured using a modification of the method proposed by *Mosedale & Parke* Change of medium was shown to be of major importance for cell survival and proliferation

The peak response was determined not only by the antigen concentration and the sensitivity of the cells but also by the culture conditions The initial response was much less influenced by the culture conditions and therefore from a technical point of view was a better measure of the cellular response

The results obtained with cultures stimulated by tuberculin in different concentrations and from persons with different degrees of tuberculin hypersensitivity suggest that the lymphocyte response may be quantitated by measuring the rate of thymidine incorporation during the first days of culture

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GEL PRECIPITATION REACTIONS BETWEEN ALKALINE EXTRACTED RUBELLA ANTIGENS AND HUMAN SERA

By

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In a preliminary report (10) we have recently demonstrated two specific gel precipitation lines with rubella antigens and rubella convalescent human sera Schmidt et al have also reported rubella gel precipitation in agarose gel (11) They were able to show at least one possibly two separate lines and the reactions were found both with viral and "soluble antigens

This report describes the gel precipitation haemagglutination inhibition and complement fixation reactions of sera from rubella patients as well as of randomly selected female sera

At least three possibly four precipitation lines specific for rubella virus were demonstrated with some of these sera

MATERIAL AND METHODS

Virus The rubella virus strain employed was the RA 7/3 strain (15) It was used at the second and third passage in this laboratory

Cells BHK 21/13 S cells in suspension culture were used in antigen preparations The details of the cell culture methods have been described elsewhere (3) The cells were used at the 11th to 30th passages in this laboratory

Preparation of antigens The methods used are reported in details by *Halonen et al* (3) For the preparation of antigens the trypsinized cells were grown in suspension in a 500 ml Erlenmeyer flask with screw cap in constant spinning with a magnetic stirrer The volume of the culture was 400 ml After a preliminary growth period of 1-2 days when the number of cells was 200-400 millions per culture the pH was adjusted to neutral with 7 per cent sodium bicarbonate and the cells were inoculated with rubella virus at a multiplicity of about 0 1 PFU/cell The cells were placed in a 35 C incubator shaken at 15 minutes intervals and after one hour the suspension was placed in a one litre flask with screw cap and 400 ml of fresh maintenance medium was added The flask with a total volume of 800 ml was now placed on a magnetic stirrer in the 35 C incubator Samples of the cells were examined once a day in a phase contrast microscope for viral changes If necessary a proper amount of sodium bicarbonate was added to change the pH to neutral After incubation for 6-7 days the culture was harvested and

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centrifuged for 30 minutes at 20000 rpm in Spinco model L ultracentrifuge using rotor no 30 A 10 per cent suspension of sediment was prepared in 0 1 M glycine NaOH buffer pH 9 0 and incubated at 35 C for 8 hours with a vigorous shaking at hourly intervals The suspension was left overnight at 4 C and the antigen was sonicated with sonicator (MSE 100 watts model) for 5 minutes Finally the antigen was centrifuged in an International centrifuge (Model PR 6 Head 253) for 10 minutes at 2000 rpm The supernatant was the gel precipitation antigen and it was stored at +4 C The procedure for preparing control antigens was identical except the cells were not inoculated with rubella virus The potency of the control antigen was tested by gel precipitation against BHK 21 antiserum prepared in rabbits

HI and CF tests Disposable microtitre U plates were used The HI method is described by Halonen et al (4) In CF tests the microtechnique of Sever (12) was used Veronal buffered diluent (VBD) with 0 1 per cent gelatine was the diluent The haemolytic system consisted of equal parts of 2 per cent sheep erythrocytes and a dilution of haemolysin containing two full units Four antigen units and two full units of complement were employed in the test After overnight incubation at +4 C the haemolytic system was added and the plates were incubated at +37 C for 1 hour Then the plates were placed at +4 C for 1-2 hours and read by visual estimation of the degree of haemolysis The inhibition of haemolysis was recorded from 4+ to — and readings 4+ and 3+ were considered positive

Gel precipitation (GP) tests The micro modification of gel double diffusion described by Wadsworth (18) and modified by Kraus et al (6) was used The gels were prepared on the 5 cm × 5 cm photographic slide glasses The matrices with steel beads in their holes were put on the agar filmed slides Four nylon threads supported the matrix Agar was allowed to flow into the 0 4 mm high reaction chamber from one of the open sides After about 30 minutes the nylon threads were carefully removed and the sides of the matrix were sealed to the slide with agar Excess agar and the steel beads were removed from the holes by suction Special Agar Noble in phosphate buffered saline (PBS) pH 7 4 was used in a concentration of 1 per cent The gels also contained 0 01 per cent sodium azide Matrix holes were filled with 25 microlitres of reactant by sterilized disposable capillary pipettes After the reactants were added the slides were placed in humidified chambers The precipitations were allowed to form at room temperature The final reading was performed after 72 to 120 hours usually 84 hours In order to make the precipitation lines more visible the slides were washed and stained First the plexiglass matrices were carefully removed and the glass slides with agar sheet were washed in PBS for 24 hours PBS solution was changed 3-4 times The slides were then stained with Amido Black (2) without drying the agar Excess stain was washed away with 2 per cent acetic acid The slides were photographed on Agfa Agepan film and the results were read on the films with a magnifying glass

Plaque titration method The method is described by Vaheri et al (16)

The sera The sera from clinical rubella virus infections were sent to our laboratory from hospitals of Turku district and some of the sera were kindly supplied by Dr Leena Vihma (17) Random sampling of normal sera was carried out from the beginning of April to the end of August 1967 The children's specimens were from patients treated in the Children's Hospital of Turku University for diseases other than rubella The adult specimens were from the maternity clinic of the University Hospital The sera were treated with kaolin and chicken erythrocytes for HI tests (4) For CF tests the sera were inactivated at 56 C for 30 minutes In the gel precipitation tests untreated serum specimens were used

RESULTS

Demonstration of Precipitating Activity in Rubella Virus Antigens

Representative results of immunodiffusion reactions between a rubella antigen and a pair of sera from a rubella patient are shown in Fig 1 Serum no 1 was taken 2 months before rubella illness and serum no 2 about four months after the illness Rubella HI and CF titres were < 10 and < 4 in the first serum and 320 and 8 respectively

TABLE 1

Rubella HI and CF Titres and Occurrence of GP Antibodies in Sera from Rubella Patients

Patient	Weeks after the onset of disease	HI titre	CF titre	Number of GP lines
V M	0	<10	<4	0
	1	160	32	0
H O	0	160	4	0
	1	160	16	0
T A	0	<10	4	0
	1	40	4	0
S K	0	<10	4	0
	1	640	4	0
M H	0	160	<4	0
	2	≥640	32	0
V P	0	80	<4	0
	2	640	32	0
H R	0	20	<4	0
	2	40	32	0
T A	0	80	4	0
	2	≥640	8	1
P T	0	10	<4	0
	3	160	16	1
S P	0	<10	<4	0
	2	≥640	16	1
	3	640	16	1
	14	≥640	16	3
	21	320	16	2
T J	0	<10	<4	0
	18	640	8	1
T M	0	<10	<4	0
	18	≥640	16	1
M S	0	<10	4	0
	25	320	16	2
	38	≥640	16	2
K A	0	<10	<4	0
	5	≥640	16	1
	15	320	8	3
	23	80	16	2
K B	0	<10	<4	0
	3	160	16	2
	8	320	32	2
	61	160	8	1
M A C.	0	<10	<4	0
	4	320	32	1
	11	320	16	1
	63	160	8	1
I J	0	<10	<4	0
	3	320	32	0
	5	320	32	1
P A	0	<10	<4	0
	7	3 0	16	1
	11	320	16	2
	26	40	N D	1
R V	0	<10	<4	0
	6	320	32	1
	17	160	[illegible]2	1

N D = not done

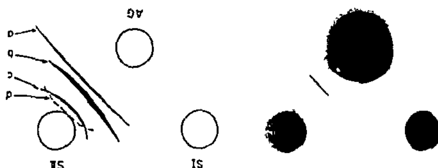


Fig 1

Immunodiffusion reactions between an alkaline extracted rubella antigen (AG) and a pair of serum specimens of a rubella patient The first serum (SI) was taken 2 months before rubella and the second serum (SII) 4 months after the illness Three clear precipitation lines are indicated by a b and c a faint line by d

in the second serum These sera exhibited no reactions with the BHK control antigens The first serum formed no precipitation line with the rubella antigen but the second serum formed at least three separate precipitation lines A fourth line (in Fig 1 line d) could be demonstrated in some experiments but it was very faint and only irregularly formed The lines a and b were easily reproducible but line c could not be found in some experiments In the ultracentrifugation of the antigen at 96000 g for 3 hours almost all GP activity remained in the supernatant only a very faint line (a) could be detected in the pellet but the whole HA activity was sedimented

TABLE 2

Relation of GP Reactions to the HI Titres in Randomly Selected Female Sera

HI titre	Number of GP positive sera 1 line	2 lines	3 lines	Number of GP negative sera	Total	% GP positive
<10	1	1	–	92	94	2
10	–	–	–	31	31	0
20	1	–		28	29	4
40	7	–	–	36	43	16
80	10	–	–	34	44	23
160	13	5	1	14	33	58
≧320	11	7		4	22	82
Total	43	13	1	39	296	19

The occurrence of GP antibodies in sera after rubella infections The HI CF and GP results of some sera from clinical rubella infections are shown in *Table 1* *The HI antibody* levels rise rapidly the CF titres more slowly and the HI and CF titres have a tendency to decrease dur

ing a longer observation period as demonstrated earlier (7 13) GP reactions were found in none of the sera during the first 10 days after the onset of illness but after two weeks 2 from 5 patients showed gel precipitation lines Almost all the sera taken 3 or more weeks after illness exhibited one or more precipitation lines The highest number of definite lines was three which were found 14 15 weeks after the onset of illness Later the number and intensity of lines showed a tendency to decrease but as long a time as 63 weeks after the illness there were still positive GP reactions (a line)

TABLE 3

Relation of GP Reactions to the CF Titres in Randomly Selected Female Sera

CF titre	Number of GP positive sera			Number of GP negative sera	Total	% GP positive
	1 line	2 lines	3 lines			
<4	–	1	–	91	92	1
4	4		–	34	38	12
8	9	1	–	13	23	43
16	8	2	1	8	19	53
≧32	3	3	–	2	8	75
Total	24	7	1	148	180	18

The occurrence of GP antibodies in randomly selected sera The GP reactions and HI titres of the randomly selected sera are indicated in Table 2 Two of the 94 HI negative sera demonstrated GP lines These sera gave however GP reactions with the control BHK antigen too Only one of the other 202 sera gave reactions with the control antigen This third serum was the only serum in Table 2 giving three GP lines One of these lines could be demonstrated with the control antigen Among the sera with low titres there were only a few showing GP antibodies to rubella About 25 per cent of sera with HI titres 80 had GP antibodies but more than 50 per cent of the sera with titre of 160 exhibited GP reactions The sera with HI titre 320 or more almost all had GP antibodies Except for one serum with titre < 10 the sera with titre 80 or lower had no more than one line All three sera having HI titres more than 640 had two GP lines About 20 per cent of all 296 sera examined had GP antibodies

In Table 3 are shown the results of the same sera correlated to CF titres Over 100 sera tested in HI were excluded because they showed anticomplementary activity More than 10 per cent of the sera having CF titres 4 showed GP reactions The sera with CF titres 8 had about 40 per cent positive GP reactions and if the titre was 32 or higher there were 75 per cent GP positive The distribution of GP reactions among the HI and CF positive sera in various age groups is shown in Table 4 It can be seen that the highest percentage of GP antibodies is

in the youngest age groups about 70 per cent. The two oldest groups have only 10–20 per cent positive rate among rubella positive sera. Only about 30 per cent of all rubella positive sera have GP antibodies when tested in this system.

TABLE 4

Relation of GP Reactions to the Different Age Groups in Rubella HI and CF Positive Sera

Age group	HI positive sera			CF positive sera		
	Number of GP positive sera	Number of GP negative sera	% GP positive	Number of GP positive sera	Number of GP negative sera	% GP positive
6–8 years	8	4	67	8	3	73
10–12 years	14	6	70	10	3	77
17–22 years	18	45	29	7	19	27
23–29 years	12	52	19	4	18	18
30–35 years	3	40	7	2	14	13
Total	55	147	27	31	57	35

DISCUSSION

The results of the present study indicate that with the technique used reproducible precipitation reactions can be obtained with all aline extracted rubella antigens. The specificity of the reactions was proved by acute and convalescent serum pairs of patients with serologically confirmed rubella and using a control BHK 21 antigen. Only 3 of the 296 serum specimens tested showed reactions with the control antigens.

Schmidt et al. (11) were able to demonstrate immunodiffusion reactions with rubella virus using agarose and chamber technique but failed in initial attempts by using agar and slide technique. However, in the present study agar gel and a modified chamber technique produced at least three specific precipitation line. The high sensitivity of this modified chamber technique has been demonstrated by *Krause et al.* (6) by detecting concentrations as low as 0.03 microgram of antigen. Later experiments in this laboratory have confirmed that agarose may be slightly better than agar. A long incubation time for the development of the precipitation lines seemed to be necessary. The first visible lines were observed after 36 hours but longer incubation times enhanced the line formation.

The results of the serum specimens collected serially from rubella patients clearly demonstrate the late development of GP antibodies. At the time of peak HI and CF titres GP antibodies were found only in a few serum specimens. The heaviest lines and the highest number of lines were demonstrated as late as 4 months after the illness. A similar late appearance of GP antibodies has been observed with the respi

ratory syncytial virus and arboviruses in immunized animals (1 5) and in experimental herpes keratoconjunctivitis (8) In this connection it is also interesting to note the very late occurrence of neutralizing antibodies to rubella virus demonstrated by *Leerhoy* (7) In the randomly selected sera the presence of GP antibodies was in good correlation with the results of HI and CF tests Practically no serum with low HI and CF titres showed GP reactions and almost all with high HI and CF titres had positive GP reactions There was also some correlation between the HI and CF titres and the number of the GP lines which increased in number when HI and CF titres were higher In the HI and CF positive sera the rate of GP positive specimens decreased in older age groups This may be correlated with the significantly higher number of higher HI and CF titres in younger age groups than in the older (9)

The antigens used in the present study were soluble They remained in the supernatant when all the haemagglutinating activity was pelleted It means that the viral particles and infectivity (14) were already at the bottom A faint activity was also demonstrated in the pellet as has been reported by *Schmidt et al* (11) The most constantly demonstrated line was the a line It was clearer and heavier than the other lines and because it was nearest to the antigen well it may be due to an antigen of larger size than the others The b line was the second from the antigen well and the c line the third The specificity of the irregular and faint d line requires further studies

An important application of the developed GP test is in the identification of rubella virus soluble antigens For the routine diagnostic work the developed test may not be *practical However* the very late appearance of GP antibody may make the gel precipitation test applicable for serological diagnosis of rubella in cases where the first specimen has been obtained several weeks after the onset of illness

SUMMARY

A gel diffusion (GP) technique using an alkaline extracted antigen was developed for rubella virus *At least* 3 virus specific precipitation lines were demonstrated The precipitating antigens were soluble only traces of these were sedimented by ultracentrifugation

The precipitating antibodies appeared two weeks or later after the onset of rash The highest number of GP lines were seen 4 months after the illness Among randomly selected sera only 20 per cent had precipitating antibodies The occurrence of GP antibodies was in correlation with the HI and CF titres More than 50 per cent of the persons having HI titre of 160 or more and CF titre of 16 or more had GP antibodies The highest percentage of GP antibodies was found in the youngest age groups

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COMBINED USE OF FLUORESCENT ANTIBODY TECHNIQUE AND CULTURE ON SELECTIVE MEDIUM FOR THE IDENTIFICATION OF *NEISSERIA GONORRHOEAE*

By

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Received 9 xii 68

In a previous study on identification of *Neisseria gonorrhoeae* (*N gonorrhoeae*) a comparison was made of the results obtained by culture and by fluorescent antibody test (FAT) (3) When FAT was performed after enrichment of the material by culturing (the delayed FAT) it was found to be slightly more sensitive than conventional culture followed by bacteriological identification The higher yield of positive results originated from the more heavily contaminated specimens especially from rectal swabs

During recent years a selective medium for isolation of gonococci was gradually developed and finally established by *Thayer & Martin* in 1964 (9) They combined the addition of ristocetin recommended by *Berger* in 1961 (1) with the addition of polymyxin B sulphate and nystatin This medium called TM medium (after *Thayer & Martin*) was found to give results superior to those obtained by *Deacon*'s delayed FAT (2) when incubation on the TM medium was carried out for 40 hours (6)

Since 1965 the Neisseria Department at Statens Seruminstitut has used a selective medium of the TM type for routine identification of *N gonorrhoeae* (11) In the present experiments the results of culture on this medium were compared with those obtained by delayed FAT after inoculation on to both selective and non selective medium

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The clinical specimens were kindly supplied by the doctors at the Venereological Out Patients Department at Rudolph Bergh s Hospital Copenhagen (Head A Perdrup MD PhD)

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MATERIALS AND METHODS

Media

Selective medium The HYL medium described by *Moller & Reyn* (7) was used but with one modification i e haemoglobin was replaced by defibrinated horse blood (12) The following antibiotics were added polymyxin B sulphate (Pfizer) 25 IU Mycostatin® (Squibb) 25 IU and Spontin® (Abbott) 10 μg/ml

Non selective medium The same medium without antibiotics

Fluorescent Antibody Test (FAT)

Preparation of rabbit antigonococcal sera and rabbit antistaphylococcal sera labelling with fluorescein isothiocyanate (FITC) and performance of the test were as described previously (3 4)

Reading ++++ denotes a brilliant yellowish green fluorescent layer covering uniformly the surface of each bacterial cell + ++ and +++ indicate increasing degrees of homogeneity and intensity of the fluorescence of a bacterial population 0 denotes a pale bluish fluorescence (barrier filter Zeiss No 47)

The specific positive reaction is characterized by the morphology of the micro organisms *in casu* typical diplococci and the degree of fluorescence (+++ and ++++)

Bacterial Strains (see Table 1)

Treatment of Specimens

Duplicate specimens were obtained from patients attending the Venereological Out Patients Department at Rudolph Berghs Hospital Copenhagen The specimens were transported to the laboratory on charcoal impregnated sterile wooden applicators in a modified Stuart medium (10) The transportation time never exceeded six hours

One swab was used for identification of gonococci by conventional culture on selective medium The gonococci were identified by Gram staining oxidase reaction and fermentation tests

The other swab was inoculated on to both selective and non selective medium the two media being used alternately After 18 hours incubation at 36 C in a moist atmosphere containing 10 per cent carbon dioxide duplicate smears were made from each plate irrespective of whether growth was visible or not The slides were fixed in methanol and stained with a mixture of FITC labelled rabbit anti gonococcal globulin and unlabelled rabbit antistaphylococcal serum (3 4) The stained smears were examined by fluorescence microscopy on the same day The corresponding plates were kept until the results obtained by conventional culture were available When culture was negative and delayed FAT positive the identity of the strain was confirmed by the usual bacteriological tests

In the case of specimens received on Saturday afternoons the procedure was slightly modified by the swab intended for routine culture was inoculated immediately but the one for FAT was kept in Stuart medium at 4 C until Monday morning and then used as described above

RESULTS

Preliminary and Supplementary Experiments

Fifty freshly isolated strains of *N gonorrhoeae* were examined by FAT after growth on both selective and non selective medium The antibiotics did not induce changes in morphology nor did they alter the stain ability of the gonococci

Various strains of saprophytic *Neisseria Mima* species *Herellea* species and *Moraxella* species plus 14 strains of *Staphylococcus aureus* were inoculated on to both selective and non selective medium On selective medium growth of all strains except some *N catarrhalis* and

TABLE 1

Growth of Various Strains on Selective and Non Selective Medium FAT Performed on Smears from Selective Medium

Strains		Growth on		FAT	
		Selective medium	Non selective medium	I	II
N. catarrhalis	ATCC 8193	1	4	++	0
N. catarrhalis	ATCC 8176	0	4	++	0
N. catarrhalis	ATCC 7900	2	4	0	0
N. catarrhalis	NCTC 4103	1	4	+	0
N. catarrhalis	SS 495/2	4	4	++	0
N. catarrhalis	SS 8/895	4	4	++	0
N. flava	ATCC 14221	1	4	+	0
N. perflava	ATCC 10555	1	4	0	0
N. subflava	ATCC 11076	0	4	0	0
N. flavescens	ATCC 13120	1	4	++	0
N. sicca	ATCC 9913	0	4	+	0
N. haemolysans	ATCC 10379	0	4	0	0
N. cuniculi	ATCC 14688	0	4	0	0
N. cuniculi var gigant	ATCC 14689	1	4	0	0
N. canis	ATCC 14687	2	4	0	0
N. caviae	ATCC 14659	0	4	0	0
N. denitrificans	ATCC 14686	0	4	+	0
Mima polymorpha	ATCC 9957	2	4	+	0
Mima polymorpha	ATCC 10373	3	4	0	0
Herellea species	ATCC 9955	2	4	+	0
Herellea species	ATCC 9954	2	4	0	0
Herellea species	ATCC 11959	2	4	0	0
Moraxella bovis	ATCC 10900	0	4	0	0
Moraxella species	ATCC 12479	1	4	0	0
S. aureus (7 reactive strains)		1	4	++++	0
S. aureus (7 non reactive strains)		1	4	0	0
N. gonorrhoeae (URI)	ATCC 11688	4	1	++++	++++
N. gonorrhoeae (J G)	ATCC 11689	4	4	++++	++++
N. gonorrhoeae (fresh control)		4	4	++++	++++

I Stainability with FITC-labelled rabbit antigonococcal globulin
II Stainability in one step inhibition test i e with a mixture of FITC labelled rabbit antigonococcal globulin and unlabelled rabbit antistaphylococcal serum cf reference No 4

one strain of *Mima polymorpha* var *oxydans* was strongly or completely inhibited (Table 1) The degree of inhibition was estimated roughly by comparison with the growth on non selective medium Macroscopically identical results were recorded as 4 and 25 per cent 50 per cent 75 per cent and 100 per cent inhibition as 3 2 1 and 0 respectively In order to obtain more quantitative information concerning the degree of inhibition a few representative strains were retested using a known inoculum followed by colony counting For instance on selective medium *Staphylococcus aureus* (growth degree 1 on selective medium) produced < 10 colonies from an inoculum of 2×10^7 cells *Mima polymorpha* ATCC 9957 (growth degree 2 on selective medium) produced < 10 colonies from 10^7 cells and *Mima polymorpha* ATCC

10793 (growth degree 3 on selective medium) produced 150 colonies from 10^4 cells Even *N catarrhalis* 163/2 (growth degree 4 on selective medium) was inhibited thirty fold

As regards all strains listed in Table 1 duplicate smears were made from the selective medium whether or not there was visible growth One smear was stained with antigonococcal conjugate diluted in saline and the other with the usual mixture of antigonococcal conjugate and unlabelled rabbit serum The antibiotics used in the selective medium did not induce any changes in stainability as compared to previous examinations (3) The two gonococcal strains ATCC 11688 and 11689 were included because they had reacted poorly with antigonococcal conjugate in a previous experiment (3) They now showed a typical specific reaction

Lucas et al (5) reported that after exposure to lethal doses of penicillin streptococci of groups A and B changed their stainability with antigonococcal conjugate Three streptococcal strains of groups A B and G were examined by FAT before and after treatment with 1000 units of penicillin per ml for 24 hours without the stainability of the strains being altered The one group A strain which was previously shown to react non specifically (3) was stained both before and after treatment with penicillin and this staining was inhibited when unlabelled rabbit serum was added to the conjugate

Finally it was examined whether the stainability of gonococci was influenced by testing with oxidase reagent (*tetramethyl p phenylene diaminehydrochloride*) Gonococcal colonies found after 24 and 48 hours incubation of primary cultures and 18 hour subcultures were examined at various times after the addition of oxidase reagent (from half an hour to 24 hours) Simultaneously a subculture was made as a test of viability The oxidase reagent neither induced changes in stainability nor killed the bacteria

Identification of N gonorrhoeae by Means of FAT after Enrichment of the Material on Selective and Non Selective Medium

Table 2 shows the results obtained by culture on selective medium followed either by bacteriological or immunofluorescent identification of the gonococci In addition the FAT was performed after inoculation on to non selective medium The material consisted of 815 duplicate specimens obtained from patients attending a venereological clinic the patients were either suspected of suffering from gonorrhoea or they were under control for the effect of treatment The results obtained by FAT from swabs which had been kept from Saturday to Monday in the icebox did not differ from the rest of the material and were therefore included without comment

The agreement between results obtained by conventional culture and by FAT was good By both methods 610 specimens were negative and

TABLE 2

Identification of N gonorrhoeae in 815 Duplicate Specimens Comparison of Culture and Delayed FAT

Culture on selective medium		FAT positive			FAT negative	Total
		Both media	Selective medium	Non selective medium	Both media	
Positive	Day 1	131	7	1	3	142
	Day ?	44	5	1	5	55
	Total	175	1?	?	8	197
Negative		4	?	2	610	618
Total		179	14	4		
		197			618	815

Day 1 = visible colonies after 18-24 hours incubation
Day ? = visible colonies after 48 hours incubation

TABLE 3

Identification of N gonorrhoeae in 175 Female Patients ([illegible] Duplicate Specimens)

	Culture	FAT positive				FAT negative	Total
		Both media	Selective medium	Non selective medium	Total	Both media	
Patients	positive	50	2	0	5?	2	54
	negative	0	0	1	1	1?0	121
	total	50	?	1	53	1??	17?
Urethra	positive	37	7	1	45	1	46
	negative	2	0	1	3	126	1 9
	total	39	7	2	48	1?7	175
Cervix	positive	45	0	0	45	4	49
	negative	0	1	0	1	119	1?0
	total	45	1	0	46	123	169
Rectum	positive	12	3	0	15	3	18
	negative	1	0	0	1	93	94
	total	13	3	0	16	96	11?
All sites	positive	94	10	1	105	8	11?
	negative	3	1	1	5	338	343
	total	97	11	2	110	346	456

189 were positive The remaining 16 specimens were distributed as follows 8 positive by culture alone and 8 by FAT alone

By culture on selective medium 197 specimens were found positive 70 per cent of which had shown visible gonococcal colonies after 24

hours incubation The remaining 30 per cent were detected after 48 hours specimens received on Saturday's afternoons included

Only one of the gonococcal strains was unable to grow on the selective medium but was detected by FAT on smears from non selective medium The strain was identified by the usual bacteriological tests and the inhibition of growth on selective medium was confirmed

As regards specimens from male patients the discrepancies in the results obtained by the different methods were negligible The results of 156 duplicate specimens from female patients are shown in Table 3 Only 13 specimens gave differing results 8 were found positive by culture alone and 5 by FAT alone The most striking figures are firstly the high degree of agreement between the results of culture and FAT in the case of rectal specimens and secondly that four cervical specimens were positive by culture and negative by FAT In all four cases very few (1-5) gonococcal colonies were found after 18 hours incubation

TABLE 4

Identification of N gonorrhoeae by FAT (1630 Duplicate Smears)

	Culture on	Both smears positive	One smear positive	Both smears negative
Males	Selective medium	85	1	273
	Non selective medium	80	5	274
Females	Selective medium	101	10	345
	Non selective medium	96	4	356
Total		362	20	1248

TABLE 5

Identification of N gonorrhoeae Comparison of FAT and Culture

FAT	1st series Culture on non selective medium 664 specimens		2nd series Culture on selective medium 815 specimens	
	Positive	Negative	Positive	Negative
Positive	173	32	189	8
Negative	5	454	8	610

Table 4 illustrates the significance of making duplicate smears from each plate for the FAT In specimens from male patients the advantage is minimal when selective medium is used for enrichment However under the same conditions 9 per cent (10/111) of the gonococci found in specimens from patients were detected in only one of the duplicate smears

Table 5 shows a comparison of results obtained by FAT and culture before and after introduction of the selective medium into our routine culture method In the first series when non selective medium was used both for bacteriological identification and for enrichment in FAT the advantage of the FAT was significant a further 13 per cent (27/210) positive results were found and these were available much earlier In the second series after the introduction of selective medium, the two methods detected the same percentage of positive results However the results of FAT were available 24–48 hours earlier than those obtained by culture

DISCUSSION

Since Deacon in 1959 introduced the use of Coons immunofluorescent technique in the diagnosis of gonorrhoea the reliability and sensitivity of this test have been confirmed by several workers In order to get results equal to those obtained by conventional culture an interposed enrichment of the material by inoculation was found necessary This fluorescent antibody test (delayed FAT) was often found to be more sensitive than culture followed by bacteriological identification even when the latter was performed under optimal conditions The selective TM medium introduced by *Thayer & Martin* in 1964 was claimed to give results superior to *Deacon's* delayed FAT when incubation on this medium was carried out for 40 hours (6) These recent developments in the laboratory diagnosis of gonorrhoea have been reviewed by *Reyn* (11) in a paper which includes a comprehensive list of references

The present experiments were performed firstly to evaluate the use of selective medium for enrichment of material to immunofluorescent identification of *N gonorrhoeae* and secondly to examine how often gonococci were inhibited by the antibiotics in a selective medium of the TM type It was demonstrated in preliminary experiments that the antibiotics used in the selective medium neither induced morphological changes in gonococci nor changed the specific stainability Furthermore the antibiotics did not induce non specific reactions of the other bacteria tested (Table 1) and these were also found to be so strongly inhibited that they only occasionally grew out after inoculation of clinical specimens The addition of oxidase reagent (*tetramethyl* p phenylene diaminehydrochloride) to primary cultures of gonococci neither altered the specific staining reaction nor killed the bacteria Thus subsequent subculturing for sensitivity determinations was not biased The latter result is not in accordance with the observations by *Peacock et al* (8) presumably because these authors used the more damaging *dimethyl* p phenylene diaminehydrochloride as reagent

The results obtained in the case of 815 duplicate specimens from patients suspected to be suffering from gonorrhoea or under control following treatment confirmed the usefulness of both methods Compari

son with a previous examination of specimens from a similar group of patients attending the same clinic (3) (Table 5) proved that the introduction of selective medium had eliminated one of the advantages of FAT, viz. the higher yield of positive results obtained from heavily contaminated specimens.

It should be mentioned that FAT has been given some handicaps in these experimental series. The swab used for bacteriological identification was streaked on to one plate, while the swab intended for FAT was used twice. Furthermore the inoculum was spread as in bacteriological procedures (10). This implies a dilution of the FAT specimen which may be particularly critical when a selective medium is used for enrichment over an 18 hour period (prolonged generation time, decreased germinating power). This is illustrated by the unexpected finding that four cervical specimens were positive by culture and negative by FAT; only one positive result was found by FAT alone (Table 3). Earlier experiments had proved the relationship to be the opposite. The same problem is reflected in the finding that about 10 per cent of the gonococci from female patients were detected in only one of the duplicate smears, thus indicating that the number of organisms present after 18 hours was still very small.

The statement by *Lucas et al.* (5) that FAT "is unsuitable for testing the efficacy of penicillin and other antibiotic therapy since residual dead bacterial forms or cross reacting organisms may be present in the specimens" could not be confirmed. Neither could it be confirmed that penicillin induces non specific staining in group A and B streptococci, at least not in the few strains tested by the author.

Only one strain was encountered which did not grow on the selective medium. The inhibitory agent was ristocetin 10 µg/ml (11). The gonococcal strains isolated in Denmark today are generally more sensitive to the antibiotics commonly used for treatment than was the case a few years ago. Since strains sensitive to less than 10 µg ristocetin tend to occur mainly among strains which are sensitive to penicillin, the proportion lost on selective medium may now be higher than at the time when the experiments reported were performed. Ristocetin has recently been replaced by vancomycin (3 µg/ml) in our routine medium. Although vancomycin seems to be less inhibitory to gonococci than ristocetin, recent investigations by *Reyn* (11) indicate that about 3 per cent of the present population of gonococcal strains are sensitive to 3 µg vancomycin/ml.

It may be concluded that ideally each specimen should be inoculated both on to selective medium for bacteriological identification and on to non selective medium for FAT, the latter possibly being performed after localization of suspicious colonies by means of the oxidase reaction. It would thus be possible both to detect a very small number of gonococci in a specimen and to detect strains that do not grow on the selective medium.

SUMMARY

A series of 815 duplicate specimens was obtained from patients attending a venereological out patients department *N gonorrhoeae* was identified by culture on selective medium of the *Thayer Martin* type and by fluorescent antibody test after enrichment of the material by culturing on both selective and non selective medium The total number of positive results was 197 Out of these 179 were positive by both methods 8 by culture alone and 8 by fluorescent antibody test alone One strain was encountered which was not able to grow on the selective medium i e it was inhibited by ristocetin (10 μg/ml)

Thus the percentage of positive results found by inoculation on to selective medium was the same whether identified bacteriologically or by the fluorescent antibody test However the results obtained by the immunofluorescent technique were available 24 48 hours earlier than those obtained from the routine procedure

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ANTIBODY INDUCED SUPPRESSION OF THE IMMUNE RESPONSE IN ANTIGEN STIMULATED CULTURES

By

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In the intact animal inhibition of the cellular and humoral immune response has been observed when specific antibodies are administered together with the stimulating antigen (*Uhr & Baumann* 1961 *Moller & Wigzell* 1965 *Wigzell* 1966) Humoral antibodies have been regarded as feed back factors regulating the cellular immune response (*Uhr & Moller* 1968) *Moller & Wigzell* (1965) and *Wigzell* (1966) have done extensive studies on the effect of γM and γG antibodies on the humoral response and the formation of cells with plaque forming ability in spleen suspensions from mice immunized against sheep red cells They presented evidence that antibody exerts an inhibitory effect on the antigen whether it is extracellular or in phagocytic cells in the spleen but found no evidence that passively administered antibodies had any direct inhibitory effect on antibody synthesizing cells

The purpose of the present work was to study the effect of antibodies on an antigen induced immunological reaction *in vitro* The effect of anti sheep red cell antibodies on cellular proliferation and specific immune response in sheep red cell stimulated cultures containing blood lymphocytes from sheep red cell immunized rabbits was investigated

MATERIAL AND METHODS

Cell Cultures

The cell cultures were prepared with lymphocytes from sheep red cell (SRC) immunized rabbits as previously described (*Lamvik* 1968 *a*) The cultures were stimulated with SRC as outlined below They were harvested after 8 days culturing apart from two culture series that were harvested after 6 days The culture medium was changed after 4 days incubation The culture medium was prepared with 4 parts of Parker s tissue culture medium (*TC199*) and 1 part of rabbit serum Each culture tube contained 2 5 × 10⁶ cells suspended in 2 5 ml of medium One half ml of TC199 with 1 per cent SRC was added to each tube as stimulator while 0 5 ml of TC199 without SRC was added to each of the control tubes

In one series of cultures rabbit serum with varying amounts of complement was used in the medium Two pooled rabbit serum samples one fresh containing com

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plement the other inactivated at 56 C for 30 min were mixed in different proportions to give a relative variation in fresh serum content in the final medium from 0 to 90 per cent

Anti SRC Antibody Sera

Two sera were tested for suppressive effect on the cellular reactions

a) One rabbit was bled 5 days after one single intravenous injection of 1 ml of 5 per cent washed SRC The serum which is called early serum showed an agglutinin titre of 64 and a lytic titre of 1024
b) One other rabbit was bled 4 weeks after the end of a 6 week immunizing course consisting of one intravenous injection of 1 ml and 14 injections of 0.5 ml of 35 per cent washed SRC The serum which is called late serum" showed an agglutinin titre of 512 and a lytic titre of 1024

The early and late sera were both diluted 1/2 1/20 and 1/200 in pooled normal rabbit serum which had been adsorbed with SRC to remove heterophil antibody (*Kabat & Mayer* 1961) The immune serum dilutions were then used in the culture experiments in two ways

In most experiments the SRC used for stimulating the cultures were pre incubated in inactivated immune serum dilutions (1 per cent cell suspension) for 1 hour at room temperature washed three times in phosphate buffered saline and suspended in TC199 to 1 per cent concentration In this way antibodies were adsorbed to the antigen in varying amounts before the antigen was added as stimulator to the culture tubes Control cultures were stimulated with SRC pre incubated in inactivated normal rabbit serum or with non incubated SRC or were cultured without stimulation Pooled adsorbed rabbit serum was used in the culture medium at the start of culturing as well as for the change of medium

In some culture experiments the immune serum dilutions were used in the culture media as the only serum addition These cultures were stimulated with normal SRC suspended in TC199 Control cultures were prepared with adsorbed and non adsorbed normal rabbit serum with and without sheep red cell stimulation At the time of medium change pooled adsorbed rabbit serum was added to all culture tubes

Cell Reaction

The cell reaction in the cultures was measured at the time of harvesting by testing for plaque forming ability in SRC monolayers in micro incubation chambers as previously described (*Lamvik* 1968 b) The total cell numbers and differential counts in the incubation chambers were determined after the addition of acridine orange stain The numbers of plaque forming cells per 10⁴ harvested cells were estimated The agglutinin titres in the culture supernatants after 4 days incubation (medium change) and at the time of harvesting were also determined

RESULTS

No definite effect of complement content on the blastoid transformation was observed in culture tubes containing varying relative amounts of fresh rabbit serum in the culture medium (Table 1) A slight but insignificant increase in the numbers of plaque forming cells was noticed concomitant with the increase in complement content However cultures without complement in the medium and without visual lysis of the erythrocytes added as stimulator still showed blastoid transformation with development of plaque forming ability Before testing cells cultured without complement in the medium for plaqueforming ability in micro incubation chambers any intact SRC still present in the culture tubes had to be eliminated by immune lysis at 37 C for

	Cultured cells	Medium	Stimulator
a	Blood lymphocytes from SRC immunized rabbits	TC 199 with normal rabbit serum	SRC incubated in immune serum diluted 1/2 1/20 1/200
b	Blood lymphocytes from SRC immunized rabbits	TC 199 with immune serum diluted 1/2 1/20 1/200	 SRC SRC SRC

Fig 1

Experimental programme for testing the effect of antibodies on the immune response *in vitro* The culture variants were checked at the time of harvesting for blastoid transformation plaque forming cells and agglutinins Control culture variants are described in the text

30 min after addition of complement Without this procedure the unlysed SRC which were coated by anti SRC antibodies present in the culture medium were transferred together with the lymphocytes to the incubation chambers and gave diffuse lysis in the sheep red cell monolayers

TABLE 1

Cellular Transformation and Numbers of Plaque Forming Cells in Lymphocyte Cultures Containing Varying Amounts of Complement

Fresh serum content of medium relative amounts %	Cellular transformation		Plaque forming cells per $10^{?}$ harvested cells
	All transformed cells %	Blastoid cell	
20	44 75±2 [illegible]	9 0 ±2 0	43 8+ 8 5
10	42 25±1 75	7 7 ±0 [illegible]	36 3±13 0
5	44 0 ±1 5	6 75±0 75	23 3± 9 1
2	38 0 ±3 5	8 5 ±0 5	24 3±11
0	37 75±4 25	7 25±1 [illegible]	24 3±11 [illegible]

Means and range of results from duplicate culture tubes

In cultures stimulated with SRC pre incubated with different amounts of early and late immune serum (Fig 1a) no suppressive effect was observed on the blastoid transformation A suppressive effect on the specific immune response was however found Fig 2 shows the results of one of several similar culture series

Late immune serum in high concentration appears to counteract

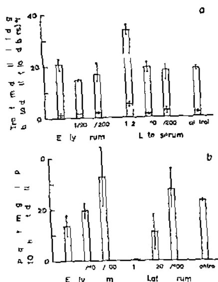


Fig 2

Cellular transformation (a) and numbers of plaque forming cells (b) in cultures containing lymphocytes from SRC immunized rabbit *In vitro* stimulator SRC pre incubated in early or late immune serum diluted 1/2 1/20 and 1/200 in normal adsorbed rabbit serum Control cultures stimulated with SRC without pre incubation Means and range of results from duplicate culture tubes after 8 days culturing

the stimulating action of SRC so that no plaque forming cells develop in the cultures Late immune serum diluted 1/20 gave a moderate reduction in the numbers of plaque forming cells compared to the effect of immune serum diluted 1/200 Early immune serum in different concentrations showed a similar effect on the stimulating ability of SRC The differences between the results following the use of different serum dilutions were however slight and probably not significant Cultures stimulated with SRC pre treated with high dilutions of immune sera gave about the same numbers of plaqueforming cells as cultures stimulated with non treated SRC

The pooled results from two other culture series are shown in Fig 3 Late immune serum was used for preincubating the SRC before addition to the culture tubes The results are compared with the results in cultures stimulated with SRC pre incubated with normal rabbit serum containing trace amounts of anti SRC antibodies Again a definite suppressive effect of late immune serum on the stimulating action of SRC on the development of plaque forming cells was found (Fig 3a) Serum diluted 1/20 had an effect intermediate between that of normal serum and immune serum diluted 1/2 The same trend was observed with the agglutinin titres in the culture supernatants (Fig 3b) In the culture tubes stimulated with SRC pre incubated with low dilutions of immune serum agglutinins in low titres were found at the time of

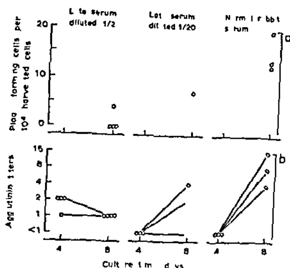


Fig 3

Numbers of plaque forming cells at harvesting and agglutinin titres at time of medium change and at harvesting in lymphocyte cultures from two SRC immunized rabbits *In vitro* stimulator SRC pre incubated in immune serum dilutions and in normal rabbit serum as indicated Eight days culturing

medium change probably due to liberation of antibodies from the SRC No increase in agglutinin titres was noticed during the last 4 days of culturing In cultures stimulated with SRC pre treated with immune serum in higher dilutions or in normal rabbit serum agglutinins appeared in the supernatants in the last days of culturing The agglutinin titres were highest in the latter culture variant

In culture series stimulated with SRC and containing varying amounts of late immune serum in the culture medium (Fig 1b) results similar to those outlined above were obtained Immune antibodies caused suppression of the development of plaque forming cells while no definite effect was observed on the degree of cellular transformation in cultures harvested on the 6th (Fig 4) as well as on the 8th day of culturing The suppressive effect on the specific immune response was usually stronger than in culture variants stimulated with SRC pre incubated in immune serum dilutions possibly due to higher antibody content in the former cultures

The supernatants in the culture variants to which immune serum in high concentrations had been added at the culture start contained agglutinins at the time of medium change also These passively added agglutinins were still present in small amounts in the supernatants after change of medium since the cells were not washed at the time of medium change Due to this contamination any slight new synthesis of agglutinins would not be detected in cultures prepared with immune serum diluted 1/2 and 1/20 In cultures prepared with immune serum in high dilution and with normal SRC adsorbed or non

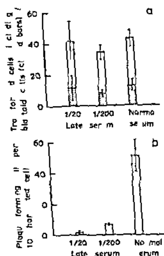


Fig 4

Cellular transformation (a) and numbers of plaque forming cells (b) in SRC stimulated cultures containing different dilutions of late immune serum in the medium compared to cultures containing normal rabbit serum Means and range of results from duplicate culture tubes after 6 days culturing

adsorbed rabbit serum agglutinins appeared between medium change and harvesting on the 8th day

DISCUSSION

The first part of this study was concerned with the possible effect of complement on the stimulating effect of SRC in blood lymphocyte cultures Culture series containing varying amounts of fresh serum demonstrated no definite effect of complement on cell transformation and development of plaque forming cells Another observation is probably related to these findings Culture medium containing SRC adsorbed serum supported the cellular reactions against SRC as did medium containing non adsorbed serum although the strongest reactions were usually found in cultures with the small amount of anti SRC lytic antibodies (heterophil antibodies) present in the pooled normal rabbit serum used in the medium with non adsorbed serum Thus the stimulating effect of SRC on the cultured primed lymphocytes is not dependent on though it may be facilitated by lysis of the added SRC

The main experiments outlined in Fig 1 concerned the possible suppressive effect of immune antibodies on the stimulating effect of SRC Late immune serum with strong SRC agglutinating ability was found to suppress the development of plaque forming cells completely and at the same time to prevent any detectable liberation of anti SRC agglutinins This latter effect could only be evaluated when immune

serum was used for pre incubation The suppressive effect was found to depend on the amount of immune antiserum used Although the estimation of plaque forming cell numbers in *micro incubation chambers gives only semi-quantitative data on the immune response (Lamvik 1968b)* the same trend was observed in all culture series

Early immune serum with high lytic and low agglutinating ability towards SRC showed in low dilutions only a weak and insignificant *suppressive effect on the development* of plaque forming cells

Late immune serum added directly to the culture medium gave the same effect as *pre incubation of the SRC in the immune serum* dilutions The antibodies added to the media would presumably be bound rapidly to the *stimulating SRC so that the final effect of the antibodies* in the cultures would be the same The suppressive effect was usually more complete when *immune serum was added to the medium* possibly due to larger amounts of antibodies applied in this way

No *suppression of cellular transformation was seen in the culture* tubes to which immune antibodies had been added despite the signs of inhibition of the *immune response which were demonstrated* The morphological evaluation of cellular transformation was done in vital preparations stained by acridine orange The harvested cells were well separated in such preparations Cell differentiation was performed without difficulties due to clear nuclear and cytoplasmatic staining in all intact cells Permanent preparations fixed and stained with May Grunwald Giemsa demonstrated also clearly the cell transformation in the cultures where the specific immune reaction had been suppressed by addition of immune serum Due to clumping of the cells accurate differential counts could not be done in such preparations however

The results show that inhibition of the complete immune response with suppression of antibody synthesis may occur without inhibition of the cell proliferation after antigen stimulation Cell proliferation normally precedes the development of antibody synthesizing cells *in vitro* as well as *in vivo* (*Baney Vazquez & Dixon 1961*) *Moller & Wigzell* (1965) found that humoral antibodies given to mice a few days after the immunizing antigen gave suppression of antibody liberation after a latency period This observation indicates that antibodies do not inhibit antibody synthesis in already committed cells They *thought that the principal in vivo* effect of the addad antibodies was to remove or counteract the stimulus for proliferation of the precursors of antibody producing cells This view is supported by the findings by *Rowley & Fitch* (1964) and *Sahiar & Schwartz (1966) that the increase in* spleen weight and the morphological changes which accompany the *primary response in vivo* is suppressed by antibodies given together with the antigen *Rowley & Fitch* found no suppressive effect of antibodies on the secondary response *in vivo* The findings in the present *in vitro* culture experiments which may be comparable to the secondary

in vivo response, suggest an inhibitory effect of the added antibodies on the specific gammaglobulin synthesis which probably occurs in the progeny of the proliferating cells without suppression of antigen induced cell proliferation The differences between the *in vitro* results and the previously reported *in vivo* findings may possibly be explained by quantitative differences in stimulating or suppressing agents The importance of the quantity of the stimulating agent is suggested by the findings by *Rowley & Fitch* (1964) that small doses of antigen induced unresponsiveness to subsequent injections of larger doses of the anti gen in contrast to the usual priming effect of larger antigen doses Antigen antibody complexes have been reported to give an increased antibody response in some *in vivo* experiments (*Uhr & Moller* 1968)

Our *in vitro* results may be explained by a change in the specificity for the induction of cell proliferation When antigen is added to the primed lymphocytes cell proliferation occurs followed by development of antibody producing cells If antigen antibody complexes are added as in the antibody inhibition experiments the complexes may possibly exert a non specific stimulating action on the lymphocytes similar to the well known non specific blastogenic agents like bean extracts and bacterial filtrates A non specific blastogenic effect of antigen antibody complexes on human blood lymphocytes has recently been reported by *Bloch Shtacher Hirschhorn & Uhr* (1967) The mechanism of specific and non specific induction of blastoid transformation is as yet un known However the present results indicate that antibodies may sup press the specific immune reaction of lymphocytes which nevertheless are able to proliferate following different types of stimulation

SUMMARY

Serum from sheep red cell immunized rabbits caused an inhibition of the immune response in cultures containing blood lymphocytes from sheep red cell immunized rabbits that were stimulated with sheep red cells A suppression of the development of plaque forming cells and the liberation of agglutinins was observed No inhibition of cellular blastoid transformation was however observed Antigen antibody com plexes seem to give rise to a non specific cellular reaction in the cul tures without the immune response which follows when pure antigen is used as stimulator

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ANTIBODY SYNTHESIS IN CULTURES OF BLOOD LYMPHOCYTES FROM TYPHOID IMMUNIZED RABBITS FOLLOWING STIMULATION IN VITRO WITH KILLED TYPHOID BACTERIA

By

J O Lamvik

Received 23 xii 68

Previous experiments (*Lamvik* 1968a 1968c) have shown that blood lymphocytes from sheep red cell (SRC) immunized rabbits were able to transform from a resting stage to specific antibody synthesis This transformation is dependent on antigen stimulation *in vitro* Antibody production was demonstrated in two ways by the presence of plaque forming cells with lytic action towards SRC and of anti SRC agglutinins in the culture medium

This report deals with the use of bacterial antigen as stimulator in cultures containing blood lymphocytes from rabbits immunized with the same bacterial antigen Fig 1 shows the experimental programme in general terms including the tests performed for signs of antibody production Gram negative entero bacteria are convenient for immunization and stimulation since they contain lipopolysaccharides easily coated on erythrocytes (*Landy, Trapani & Clark* 1955) which then may be used for testing the cultured cells for plaque forming ability and the culture supernatants for liberated agglutinins

MATERIAL AND METHODS

Antigens

Salmonella typhosa killed by heat at 60 C for 1 hour was used for *in vivo* immunization and *in vitro* stimulation *Lipopolysaccharide* from *S typhosa* was obtained from Difco Detroit Mich The lipopolysaccharide (code 3946) prepared by Boivin extraction was coated to SRC by one of the methods described by *Landy Trapani & Clark* (1955) The material was treated with alkali (0 02N NaOH in saline) at 37 C for 12 h Following neutralization with HCl a solution containing 10 µg of lipopolysaccharide per ml was prepared Washed SRC were made up to a 1 per cent suspension in saline mixed in equal volumes with the lipopolysaccharide solution and incubated at 37 C for 2 hours followed by washing three times in saline

This work was supported by a grant from the Norwegian Cancer Society The suspension of heat killed *S typhosa* was kindly prepared by Mrs. Gunvor Fretheim the Gade Institute Department of Microbiology

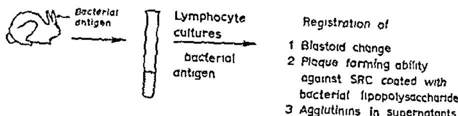


Fig 1

Schematic drawing with an outline of the experimental programme

Immunization

Four adult albino rabbits were immunized against *S typhosa* First 0.5 ml of saline suspension containing 10 bacteria was given intravenously followed by 1 ml (2×10^7 bacteria) twice weekly for four weeks Thereafter one injection weekly was given for 6 weeks followed by four biweekly doses Three booster-doses were then given in the course of one week and the rabbits were bled 5 or 6 days after the last dose At the time of bleeding the serum agglutinin titres against *S typhosa* lipopolysaccharide coated SRC varied from 1000 to 8000 Lytic antibodies were found in titres from 500 to 4000 No agglutinins and lytic antibodies (anti SRC) in low titres only were found in the sera from the control rabbits

Culture Technique

Cell suspensions for culturing were prepared as previously described (Lamvik 1968 a) from the four immunized rabbits and from two control ones Each culture tube contained 2.5×10^6 cells in 2.5 ml of medium composed of Parker's tissue culture medium (TC199) with 20 per cent pooled normal rabbit serum The culture tubes were stimulated at the start with heat killed *S typhosa* in doses ranging from 10^9 to 10 bacteria suspended in 0.5 ml of TC199 Duplicate or quadruplicate tubes were stimulated with each dose Duplicate culture tubes were supplied with 0.5 ml of TC199 without stimulator Some culture tubes were harvested after 5 days incubation Others were cultured for 8 days with a change of medium on the fourth day

Tests for Immune Reaction

The cells used for culturing from immunized and non immunized rabbits were tested before incubation for plaque forming ability against SRC coated with *S typhosa* lipopolysaccharide as described below At the time of harvesting the culture supernatants were separated from the cells and stored at -20 C and later checked for agglutinins against SRC coated with lipopolysaccharide The harvested cells were washed once in 5 ml of Ringer solution and suspended in 0.2 ml of TC199 The suspended cells were then mixed in equal volumes with a 20 per cent suspension of SRC coated with lipopolysaccharide with 20 per cent fresh guinea pig serum in TC199 The mixtures were tested in micro incubation chambers for the formation of lytic plaques in the mono layers of SRC formed on the bottom of the chambers (Lamvik 1968 b)

The degree of cell transformation in the culture was estimated in acridine orange stained cell suspensions transferred to incubation chambers The cells were classed as small lymphocytes intermediate cells and blastoid cells according to morphological criteria defined previously (Lamvik 1968 b) The second group probably comprised partly transformed lymphocytes as well as some of the progeny of blastoid cells including some cells with a morphological appearance like that of plasma cells Permanent cell preparations were made in a sedimentation apparatus (Bøe, Wendt & Schreiberg 1964) fixed and stained with May Grunwald Giemsa

The culture supernatants from the time of harvesting and from the change of medium were tested for agglutinins by titration against lipopolysaccharide coated SRC (1 per cent in saline) either directly or following concentration to 1/3 volume using polyethylene glycol The titrations were performed in Perspex trays and read from the settling pattern

RESULTS

No plaque forming cells and no agglutinins against lipopolysaccharide coated SRC were found in the cell suspensions prepared from immunized and non immunized rabbits Less than 5 per cent partly transformed cells and no blastoid cells were found in these cell suspensions After four days culturing when the medium was changed in tubes which were harvested after 8 days there were still no agglutinins present

When the culture tubes were stimulated with 10^6 or 10^8 killed bacteria and harvested after 5 days many transformed cells including blastoid cells were found in the cultures containing cells from the immunized rabbits A bacterial dose of 10^4 or less gave no definite morphological response Despite signs of blastoid transformation in the cultures harvested after 5 days incubation only very few plaque forming cells were found No agglutinins were present in the culture supernatants

In the culture tubes harvested after 8 days incubation a clear difference was observed between non stimulated and antigen stimulated cultures of cells from immunized rabbits The response towards stimulation was dose dependent From 25 to 40 per cent transformed cells including from 3 to 12 per cent blastoid cells were found in the culture tubes stimulated with 10^8 or 10^6 killed bacteria (Table 1) When the stimulating dose was 10^4 or less the response was slight with 10 to 15 per cent enlarged lymphocytes without definite blastoid change The non stimulated cultures contained no blastoid cells but some partly transformed cells were present

TABLE 1

Blastoid Cells in Antigen Stimulated Cultures Containing Blood Lymphocytes from S typhosa Immunized and Non Immunized Rabbits, Harvested after 8 Days Culturing

Stimulating dose		Blastoid cells %				
		10^5	10^6	10^4	10	None
Immunized rabbits	1		3 0	0 25	0 5	0
	2		3 75	0	0	0
	3	6 0	3 5	0		0
	4	11 5	5 0	0		0
Non immunized rabbits	1		0 75	0	0	0
	2	0	0	0	0	0

Means of result from duplicate culture tubes

Plaque forming cells (Fig 2) were found in all culture tubes containing cells from immunized rabbits harvested after 8 days when a stimulating dose of 10^8 bacteria had been added at the start of culturing (Table 2) No plaque forming cells were present in non stimulated cultures or in the cultures of cells from non immunized rabbits

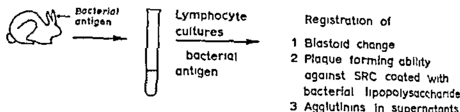


Fig 1

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Tests for Immune Reaction

The cells used for culturing from immunized and non immunized rabbits were tested before incubation for plaque forming ability *against* SRC coated with *S typhosa lipopolysaccharide as described below* At the time of harvesting the culture supernatants were separated from the cells and *stored at* —20 C *and* later checl ed for agglutinins a ainst SRC coated with lipopolysaccharide The harvested cells were washed once in 5 ml of Ringer solution and suspended in 0 2 ml of TC199 The suspended cells were then mixed in qual *volumes* with a 20 *per cent* suspension of SRC coated with lipopolysaccharide with 20 per cent fresh guinea pig serum in TC199 The mixtures were tested in *micro incubation* chambers *for the* formation of lytic plaques in the mono layers of SRC formed on the *bottom of the* chambers (*I amoil* 1968 b)

The degree of cell transformation in the cultures was estimated *in acridine* orange stained cell suspensions transferred to *incubation* chambers The cells were classed as small lymphocytes intermediate cells and *blastoid cells* according to morphological criteria defined previously (*Tamoik 1968 b*) *The second* group probably comprised partly transformed lymphocytes as well as some of the progeny of bla toid cells including some cells with a morphological appearance like that of pla ma cells Permanent cell preparations were ma le in a sedimentation apparatus (*Bots W nt & Schaberg* 1964) fixed and stained with May Grunwald Giemsa

The culture supernatants from *the time* of harvesting and *from* the change of medium were tested for a glutinins by titration against lipopolysaccharide coated SRC (1 per cent in saline) either directly or following concentration to 1/3 volume using polyethylene glycol The *titrations* were performed in Perspex trays and read *from the settling pattern*

incubation chambers where they aggregated around possible antibody liberating cells and seemed to prevent these antibodies from diffusing out into the red cell monolayers thus preventing plaque formation

The appearance of agglutinins in the culture supernatants correlates well with the development of plaqueforming cells as agglutinins (titres up to 4) were only found in the cultures of lymphocytes from immunized rabbits stimulated with an antigen dose of 10^6 bacteria Supernatants concentrated 1/3 showed a higher agglutinating ability corresponding to the increase in protein concentration (Table 3) No agglutinins were detected in concentrated supernatants from control culture series or in the supernatants from the reacting culture tubes after 4 days incubation

TABLE 3

Agglutinins Against SRC Coated with S typhosa Lipopolysaccharide in Concentrated Culture Supernatants from Antigen Stimulated Cultures Containing Blood Lymphocytes from Immunized and Non Immunized Rabbits Stimulating Dose 10^6 Killed Bacteria

Culture time		Agglutinin titres	
		4 days	8 days
	1	<1	2
Immunized	2	<1	4
rabbits	3	<1	2
	4	<1	8
Non immunized	1	<1	<1
rabbits	2	<1	<1

Supernatants from duplicate culture tubes were pooled and concentrated to 1/3 volume using polyethylene glycol

Cultures showing plaque forming cells or agglutinins against lipopolysaccharide coated SRC were checked for plaque forming ability and agglutinins against SRC with negative results

DISCUSSION

The present experiments demonstrate the development of specific reacting plaque forming cells and agglutinins in blood lymphocyte cultures following in vitro stimulation with bacterial antigen The rabbits that supplied the lymphocytes were bled 5 or 6 days after the last of three booster injections following a long course of immunization No plaque forming cells were found however in the cell suspensions used for culturing

The culture experiments showed that cell transformation with blastoid cells and some cells like plasma cells occurred when the cultures were stimulated with the priming antigen No such transformation was seen when the antigen was added to cultures of non primed

lymphocytes Cells with specific lytic activity against antigen coated SRC were found in the antigen stimulated cultures of primed lymphocyte after 8 days culturing and agglutinins against similarly treated SRC were present in the culture supernatants The development of plaque forming cells as well as agglutinins is thus dependent on *in vivo* priming and *in vitro* antigen stimulation

The numbers as well as the size of the lytic plaques found in the test chambers were smaller than the numbers and size of the plaques previously registered in most culture experiments using lymphocytes from SRC immunized rabbits (*Jamvil* 1968c) The small size of the plaques is comparable to the findings reported by *Cunningham Smith & Mercer* (1966) who used the same technique for testing cells from sheep lymph nodes and efferent lymph for plaqueforming ability against SRC coated with *S munchen lipopolysaccharide* The agglutinins found in our cultures were also present in very low titres in *some culture tubes* detectable only in the undiluted supernatant The main reason for these low titres is probably that the *in vitro* conditions for cell growth and differentiation are inferior to those in the lymphoid organs The low titres may in addition partly be explained by low cell numbers and relatively large volumes in the culture tubes

The immune reaction found in our cultures is clearly different from the spontaneous *in vitro* liberation of antibodies from peripheral blood leucocytes reported by *Landy et al* (1964) and by *Hulliger & Sorkin* (1963 1965) This antibody liberation from leucocytes without antigen stimulation *in vitro* is probably due to the escape of antibody producing cells from the lymphoid organs into the peripheral blood The results also appear to differ from those obtained by *Wesslen* (1952) and by *Hallander & Danielsson* (1962) on the liberation of antibodies against *S typhosa* and horse serum from thoracic duct lymphocytes taken from rabbits after immunization and incubated *in vitro* without *in vitro* stimulation No evolution of plaque forming cells or liberation of agglutinins was noticed in our cultures without *in vitro stimulation*

In the present experiments the signs of specific antibody liberation developed between the 4th and the 8th day of culturing Correlated with the lack of immune reaction in non stimulated cultures this evidence is indicative of *in vitro* formation of antibody producing and liberating cells in the cultures and the evolution of such cells from resting lymphocytes that lack the ability to develop spontaneously into antibody producing cells

The results are closely similar to the findings in SRC stimulated cultures with blood lymphocytes from SRC immunized rabbits (*Jamvik* 1968c) and appear to be similar to some of the findings reported by *Girard* (1968) on antibody synthesis by rabbit *blood lymphocytes in vitro* following stimulation by different antigens Bacterial antigens as well as heterologous red cells are thus able to induce cell transformation with specific antibody synthesis in rabbit blood lymphocyte cul

tures The results also show that the indirect method of registration of plaque forming ability using red cells coated with bacterial lipo polysaccharides is suitable for testing cultured lymphocytes for spe cific immune reaction

SUMMARY

Blastoid transformation was demonstrated in cell cultures containing blood lymphocytes from typhoid immunized rabbits stimulated *in vitro* with killed *S typhosa* Plaque formin cells and liberated agglutinins against SRC coated with lipopolysaccharide from *S typhosa* were found in such cultures The blastoid response as well as the specific immune respon e was dependent on the stimulating dose given *in vitro* However although a strong blastoid response occurred following a very large bacterial dose this was not followed by the appearance of plaque forming cells and agglutinins possibly because the transformed cells became coated by the excess bacteria

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IMMUNODIFFUSION STUDIES ON ESCHERICHIA COLI

1 *Identification of O, K and H Antigens in an O6 Strain*

By

JAN HOLMGREN GÖSTA EGGERTSEN LARS Å HANSON
and KNUT LINCOLN

Received 2 1 69

In 1943 *Kauffmann* reported that after elimination of heat labile envelope antigens *E coli* strains could be grouped with agglutinating antisera against the heat stable somatic antigens (20) *This provided* the key to the antigenic analysis of *Escherichia* The classical coli serology reviewed by *e g Kauffmann* (21) is based on three antigens designated O K and H The thermostable cell wall O antigens labelled O1 through O149 are well characterized lipopolysaccharides (26 31) Among the K antigens there is a distinction between L A and B antigens The L antigens are thermolabile *i e* destroyed by boiling while the B antigens lose their immunogenicity but retain their agglutinin fixing ability after such heat treatment The A antigens are thermo stable capsular antigens However these differences between the K antigens are not always quite distinct (37) The A and B antigens investigated so far have been found to consist of acid polysaccharides (18 29)) whereas at least some L antigens have been demonstrated to be proteins (29 34) The flagellar H antigens are of a protein nature (1 40)

Much of the work on the somatic antigens of Gram negative bacteria has been done on *Salmonella* Immunodiffusion techniques have had extensive application to *Salmonella* (*i a* 4 16 23 33 35 36 42) but such studies on *Escherichia* are not equally numerous (2 9 10 11 12 19 27, 29 34 35) Most of these investigations have been particularly concerned with the degree of heterogeneity of various bacterial extracts

The purpose of the present study was to obtain a more complete picture of the complex antigenic pattern of *E coli* than that given in the antigenic formula of *Kauffmann Knipschildt Vahlne* (21) and to

Thanks are due to Miss *Birgitta Berglind* Mrs *Brita Drews* and Miss *Ingela Svardstrom* for skilful technical assistance
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investigate the possibilities of a differentiation between the O K and H antigens by means of immunodiffusion methods. This characterization of E coli antigens is part of a study of the bacteriology and immunology of urinary tract infections in children (3 13 17 43 44)

MATERIAL AND METHODS

Bacterial Strains

The following *E coli* type strains kindly supplied by Drs *I & F Ørskov* at the WHO International Escherichia Centre Statens Seruminstitut Copenhagen were used

Seruminstitut designation	Serotype O	K	H
U5/41	1	1	7
U9/41	2	1	4
U4/41	4	3	5
Bi 7458/41	6	2a 2c	1
Su 4344/41	6	13	1
Bi 7509/41	7	1	–
G 3404/41	8	8	4
Su 4411/41	14	7	–
F 10018/41	18	76	14
F 14a	22	13	1
E 3b	75	?	5

In addition 29 *E coli* strains isolated from children with urinary tract infections were employed. All of these were O grouped as described by *Lincoln* (24). The O antigen groups O1 O4 O6 O7 and O18 were each represented by four strains O75 by three strains and O3 O11 O2? O25 O11? and O120 by one strain each

All the aforementioned strains were of the smooth colony type. From the O18 K76 H14 strain we isolated a rough colony variant designated O18 R which was also employed

E coli O6 K2a 2c H1 was chosen as a *model strain* for the antigenic analyses since it belongs to an O group very often found in patients with urinary tract infections (43) and is a serologically well defined strain (21 37) with no tendency to convert from the S to the R form.

Antigenic Preparations for Immunodiffusion Analyses

Stock suspensions were *prepared* in the following way: smooth colonies the bacteria of which showed no auto agglutination on heating were selected as inocula. The bacteria were cultivated on 0.7 per cent nutrient agar plates incubated for 18 24 hours at 37 C. The growth was suspended in sterile saline. The stock suspensions were adjusted to correspond to 50 mg of acetone dried bacteria per ml

Veronal buffer extract (VE antigen). Five g of acetone dried bacteria were suspended in 100 ml of veronal buffer pH 8.6 and kept at 37 C for 24 hours. After centrifugation (3000 rpm 30 min) the supernate was decanted and used as antigen. This method for preparing antigen is known not to denature proteins (4). VE antigen was prepared from the model strain (O6 K2a 2c H1) and from the O? K1 H4 O K13 H1 O14 K7 H– and O?? K13 H1 strains

Ultrasonic extract The stock suspension of the model strain (O6 K2a 2c H1) was treated for 15 min at 500 W and 20 kc/sec in an MSE ultrasonic disintegrator (MSE Ltd London). The supernate obtained by centrifugation was used

Free e press extract was prepared from the stock suspension of the model strain

(O6 K2a 2c H1) as described by *Edebo* (7) The bacterial debris was spun down and the supernate used as antigen

Heat extract antigen (HA) The stock suspension was heated at 100 C for 2 hours After centrifugation the supernate was used as antigen HA preparations were made from each of the aforementioned strains

Purified lipopolysaccharides (LPS) prepared according to *Westphal et al* (41) from *E coli* O1 O2 O4 O6 O7 O8 O18 and O75 were also used and are referred to as O1 LPS O2 LPS etc These preparations were kindly supplied by *Drs B & K Jann* at the Max Planck Institut für Immunbiologie Freiburg In the immuno diffusion studies the preparations were employed at a concentration of about 1 mg/ml

Antigenic Preparations for Immunization

Three types of antigenic preparations were used for immunization live formalin killed and heated cultures of bacteria The cultivations were performed in an anti gen free medium (15) at 37° C for 6–8 hours attaining a density of 200–500 millions of bacteria/ml (viable count) The bacteria were killed by adding formalin to the cultures to a concentration of 0 5 per cent or by boiling the cultures in a water bath for two hours (heated cultures)

Antisera

Antisera were produced in rabbits weighing 2 3 kg by two series of intravenous injections of antigenic preparation The injection volumes in ml in the first series were 0 25 0 5 1 0 and 1 0 in the second 1 0 2 0 4 0 4 0 and 4 0 all given at five day intervals During the course of the immunizations the animals were bled each week and a final bleeding was taken at sacrifice ten days after the last injection

OKH antisera against *E coli* O6 K2a 2 H1 and O14 K7 H– were produced using formalin killed cultures for the first series of injections followed by injections of live cultures in the second

O antisera were produced by injecting heated cultures in both immunization series against the typed strains of *E coli* belonging to O groups 1 2 4 6 7 8 18 and 75

RKH antiserum was produced in the same way as OKH antisera except that formalin killed and live cultures of the R variant of O18 K76 H14 (O18 R) were used

Immunodiffusion Methods

Immunological analyses were performed with the microplate double diffusion in gel technique described by *Wadsworth* (38) and with the immunoelectrophoretic and comparative immunoelectrophoretic techniques in the modifications of *Wadsworth & Hanson* (39) The electrophoretic separation was performed at a voltage of 5 V/cm during 80 min in 0 05 M veronal buffer pH 8 2 The results of the various antigenic analyses are based upon replicate experiments using analogous antisera from different rabbits as well as different bleedings from the same animal

As regards O grouping of *E coli* strains a simplified immunodiffusion method was employed Paper discs soaked with different O antisera were placed on a 2 mm thick agar layer in which basins were cut and filled with heat extract antigens of the strains to be grouped (cf 6 8 34 and experiment shown in Fig 8)

Titration of Agglutinins to E coli was performed as described by *Lincoln* (14)

Block Electrophoresis

Horizontal block electrophoresis was performed on the VF antigen from *E coli* O6 K2a 2c H1 using Sephadex G–25 (Pharmacia Uppsala Sweden) as supporting medium A slurry was made of the Sephadex with veronal buffer (pH 8 6 μ 0 075) and spread 3–5 mm thick on a glass plate (36 × 12 cm) Thereafter the antigen was applied in a 5 × 90 mm transverse basin cut in the gel The electrophoretic run was performed at a voltage of 6 V/cm for 16 hours whereupon the block was cut transversely in 3 cm strips which were eluted with the veronal buffer The fractions were tested by double diffusion and comparative immunoelectrophoresis The

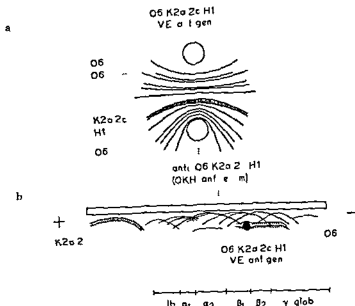


Fig 1

Schematic diagram of double diffusion (a) and immunoelectrophoretic (b) analyses of the model strain VE antigen developed with OKH antisera Lines formed by O K and H precipitinogens identified by the experiments described in this paper are indicated The electrophoretic distribution of human serum proteins is included as a reference

carbohydrate content of the fractions was determined by the anthrone reaction (14) with dextran as standard Protein was determined with the *Folin Ciocalteau* reaction (25) with tyrosine as standard

RESULTS

The Antigenic Complexity of the Model Strain

By means of comparative immunological analyses of various antigenic preparations i e veronal buffer extract (VE antigen) ultrasonic extract freeze press extract and heat extract antigen (HA) the model *E coli* strain (O6 K2a 2c H1) was shown to contain more than 20 separate antigenic factors The VE antigen was found to be the most representative antigenic preparation containing the largest number of demonstrable precipitinogens and was therefore chosen for the detailed analyses In this antigen at least 12 separate precipitating factors (Fig 1a) were revealed by comparative double diffusion analyses using different corresponding OKH antisera and other antigenic preparations from the model strain Immunoelectrophoretic studies demonstrated a good electrophoretic separation of the factors resolving the VE antigen into a total of at least 14–15 precipitinogens (Fig 1b) Some of the OKH antisera against the model strain lacked demonstrable antibodies to one or a few of these antigenic factors

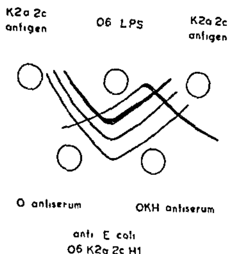


Fig 2

Schematic representation of comparative double diffusion precipitation patterns formed by the O6 LPS and the isolated K2a 2c antigens from *E coli* O6 K2a 2c H1 with an O and an OKH antiserum against *E coli* O6 K2a 2c H1

Separated
O6 LPS



anti O6
(O antiserum)

Fig 3

Immunoelectrophoretic pattern typical of those formed by the electroseparated *E coli* LPS antigens and their corresponding O antisera

Identification of O, K and H Antigens in the Model Strain

The O antigen When analysed with O6 O antisera by double diffusion three heat stable antigenic factors were demonstrable in the purified O6 LPS antigen (Fig 2) By comparative double diffusion these factors could also be demonstrated in the VE antigen from the model O6 strain using corresponding O or OKH antisera cf Fig 1a In immunoelectrophoresis the electroseparated O6 LPS developed with O6 O antisera a broad fuzzy precipitate close to the antigen basin (Fig 3) In the case of some antisera this precipitate consisted of at least two parallel lines The O6 LPS only reacted with O antisera against *E coli* O6 and not with any of the O antisera against the O groups 1 2 4 7 8 18 or 75

Experiments with the purpose of identifying the O antigen in the VE antigen preparation from the model strain were also made in which the strains of the serotypes O6 K2a 2c H1 O6 K13 H1 and O22 K13 H1 were compared with OKH antisera to O6 K2a 2c H1 Fig 4 shows two antigenic factors common to the two O6 strains

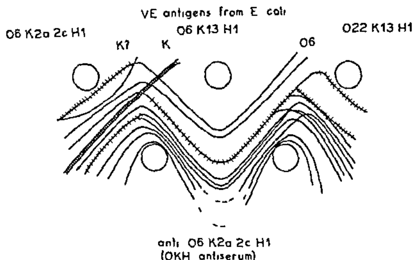


Fig 4

mparative double diffusion analysis identifying the K and O precipitinogens in e VE antigen preparation of the model train with an OKH antiserum against this rain The third O precipitinogen s e Fig 1a was not developed with the employed antiserum

which are not present in the O22 strain Comparative double diffusion analysis of O6 LPS and VE antigen from the model strain showed hat these two factors corresponded to two of the O6 factors indicated n Fig 1a

The K antigen The comparison between the strains O6 K2a 2c H1 O6 K13 H1 and O22 K13 H1 in immunodiffusion with anti O6 K2a 2c H1 OKH immune serum illustrated in Fig 4 was used to identify the K2a 2c antigen Two lines formed with the model strain O6 K2a 2c H1 were not found to be formed with the strain O6 K13 H1 which according to the *Kauffmann Knipschildt Vahlne* antigenic formula only differs from the model strain in K antigen Another experiment with a view to identifying the K antigen was performed in which the anti O6 K2a 2c H1 immune serum was absorbed with VE antigen prepared from the strain O6 K13 H1 This absorbed antiserum should only contain antibodies to the K2a 2c antigen The VE antigen from the model strain (no 4 basin in Fig 5) was analysed with the anti O6 K2a 2c H1 OKH immune serum (no 1 basins) and with the absorbed antiserum anti K2a 2c (no 2 basins) The figure shows that only one dense precipitate possibly consisting of two lines is formed with this absorbed serum In immunoelectrophoresis the VE antigen from the model strain gave a broad and dense precipitate with the "anti K2a 2c serum This precipitate often consisting of two parallel lines was formed by the fastest moving antigenic factor (Fig 1b) The high electrophoretic mobility of this factor allowed the isolation of

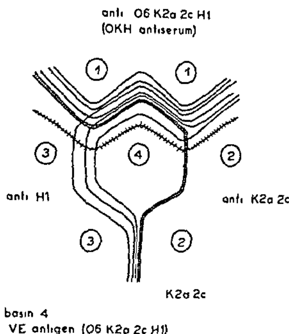


Fig 5

Immunodiffusion analysis identifying the K (right side) and thermolabile (left side) precipitinogens in VE antigen from the model strain (basin no 4) by means of anti O6 K2a 2c H1 (no 1 basins) and absorbed antisera In no 2 basins anti K2a 2c = anti O6 K2a 2c H1 absorbed with VE antigen from O6 K13 H1 In no 3 basins anti H1 = anti O6 K2a 2 H1 absorbed with HA from O6 K2a 2c H1

K2a 2c antigen uncontaminated by other antigenic material by preparative zone electrophoresis in Sephadex G-25 The K antigen containing fraction was rich in carbohydrate and could be freed from protein by i a precipitation with (NH_4) SO After heating to 100 C for 2 hours the K antigen was still precipitinogenic and formed two lines with O6 K2a 2c H1 OKH antisera but no lines with O antisera By double diffusion analysis of *E coli* O6 LPS with OKH antisera against the model strain the K2a 2c antigen could be identified as a fourth factor in the O6 LPS in addition to the three factors demonstrable with O antisera (Fig 2)

The H antigen The comparison between the three strains differing in O and K antigens but having the same H antigen shown in Fig 4 illustrates that these strains have many antigenic factors in common one or more of which can be of H antigenic nature At least ten such factors were observed some eight of which lost their antigenicity after heating to 100° C for 2 hours Most of these common factors were also found in strains with other H designations (*e g* *E coli* O2 K1 H4 and O14 K7 H–)

In an effort to identify the H1 antigen the OKH antiserum to O6 K2a 2c H1 was absorbed with HA from the same strain Analysis of the VE

antigen from the model strain (no 4 basin in Fig 5) using this absorbed antiserum (no 3 basins) showed at least three antigenic factors In an attempt to making the absorbed antiserum monospecific for the H1 antigen it was further absorbed with VF antigen from the cross reacting strains O2 K1 H4 and O14 K7 H After these absorptions the antiserum formed only one line with the VF antigen from the model strain This line was presumably formed by the H1 antigen (Fig 6)

Identification of O Antigens in Other E Coli *Strains*

Immunoelectrophoretic analysis of LPS preparations from *E coli* of the O groups 1 2 4 7 8 18 and 75 with their respective O antisera showed broad precipitates of the same form and localization as the precipitate obtained with the O6 LPS and O6 O antiserum shown in Fig 3 With the exception of LPS from *E coli* O18 and O4 which cross reacted (Fig 7a and b) the LPS antigens only reacted with their matching O antisera

TABLE 1

O Grouping of 29 E coli Strains Using O Antisera against Type Strains in the Simplified Immunodiffusion Technique

E coli HA preparation		O antiserum							
O group	No of strains	O1	O2	O4	O6	O7	O8	O18	O75
O1	4	++	—	—	—	—	—	—	—
O4	4	—	—	++	—	—	—	+	—
O6	4	—	—	—	++	—	—	—	—
O7	4	—	—	—	—	++	—	—	—
O18	4	—	—	+	—	—	—	++	—
O75	3	—	—	—	—	—	—	—	++
O3	1	—	—	—		—	—	—	—
O11	1	—	—	—	—	—	—	—	—
O22	1	—	—	—	—	—	—	—	—
O25	1	—	—	—	—	+	—	—	—
O112	1	—	—	—	—	—	—	—	—
O120	1	—	—	—	—	—	—	—	—

+ indicates faint precipitate ++ indicates dense precipitate

Further experiments with a view to establishing the O antigen specificity of precipitinogens from *E coli* were performed with the 29 O grouped *E coli* strains isolated from patients with urinary tract infections see Table 1 HA preparations from these strains of known O groups were tested with the eight O antisera anti O1 O2 O4 O6 O7 O8 O18 and O75 using the simplified immunodiffusion method (Fig 8) One dense precipitate was formed by the HA preparation from each strain and its matching O antiserum In addition the four O4 and the four O18 strains mutually cross reacted the heterologous precipitate however being fainter than the homologous These cross reactions

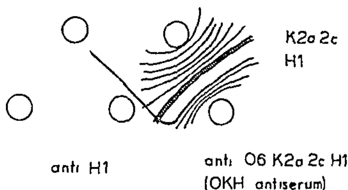
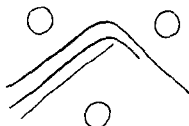


Fig 6

Comparative double diffusion analysis identifying the H1 precipitinogen in the VE antigen from the model strain using anti O6 K2a 2c H1 and this immune serum absorbed with the HA preparation from the model strain as well as with VE antigens from *E coli* O14 K7 H– and *E coli* O2 K1 H4 The triple absorbed antiserum is indicated anti H1

a



b

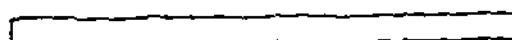


Fig 7

Comparative double diffusion (a) and immunoelectrophoretic (b) analyses showing cross reactivity of the *E coli* O4 and O18 LPS antigens using O antiserum against *E coli* O4 K3 H5

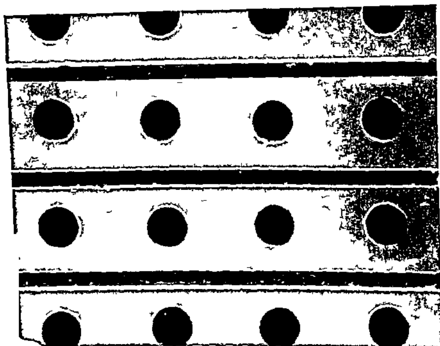


Fig 8

Photograph of analyses with the disk immunodiffusion technique for O grouping. Disks saturated with O antiserum against the O antigens from left to right O1 O2 O4 O6 (first and third rows) and O7 O8 O18 O75 (second and fourth rows). Longitudinal basins filled with HA preparations from *E. coli* strains belonging to O75 (upper basin), O7 (middle basin) and O6 (lower basin)

were verified in microplates by comparisons with the homologous systems as shown in Fig. 7a. A precipitate was also formed by the O25 strain and the anti O7 O antiserum. In comparative double diffusion analyses using the microplate method this precipitate was shown to give a reaction of fusion with one of the two lines formed by the O7 LPS and the anti O7 O antiserum. On testing for cross agglutination the O25 strain was positive with the O7 antiserum at a dilution of 1:400, whereas the O7 strain was agglutinated at dilution 1:3,200 of this antiserum. Cross agglutination between the O4 and O18 strains was also obtained.

The HA preparations from the 29 *E. coli* strains were also tested with the same O antisera in microplates with results corroborating those in Table 1. For practical reasons the anti O2 and O8 O antisera were excluded in these tests.

Comparison of S and R Variants

Another experiment with the purpose of identifying the O antigen of *E. coli* was performed in which the S and R forms of O18:K76:H14

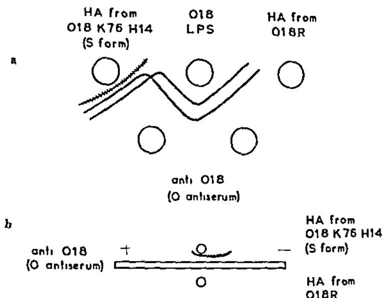


Fig 9

Comparative double diffusion (a) and immunoelectrophoretic (b) analyses showing the difference in precipitinogenic content in HA from the smooth and rough forms of *E coli* O18 K76 H14 The O antigen was not demonstrable in the preparation from the rough form

were compared Immunodiffusion analyses with O18 O antisera showed the presence of three heat stable antigenic factors in the S form of this strain Two of these factors were also present in the O18 LPS antigen but were not found in the HA preparation from the O18R strain (Fig 9a) Immunoelectrophoretic studies using the HA preparations of the S and R forms of the O18 strain with several O18 O antisera confirmed this finding a characteristic broad precipitate close to the antigen basin was developed by the S form while no line was found with the R variant (Fig 9b) Antiserum to the O18R variant (RKII antiserum) did not form any precipitate with the HA preparation of the S form

DISCUSSION

Through extensive studies using bacterial agglutination the *Escherichia* genus is serologically well defined by its O K and H antigens (21) Among the many antigenic factors revealed in the present studies of a model *E coli* strain (O6) by immunodiffusion methods some could be identified as related to O K and H antigens In addition several other antigens were common in strains which differed from the model strain in their O K and H antigens Presumably none of these common antigens corresponds to the common antigen of *Kunin Beard and Halmaggi* since the latter according to reports is not precipitinogenic (22) The M antigen (*11 18 30*) *might* be one of the

common antigens since it has been found to be antigenically identical in several different strains (32)

In various ways it could be demonstrated that the O6 model strain comprised precipitinogens which correspond to the O6 lipopolysaccharide In immunoelectrophoresis the O antigen appeared as a broad and dense precipitate in the region corresponding to that of the human serum β_2 globulin region The form of the precipitate indicates a poorly diffusing antigen of high molecular weight (Fig 1b) This precipitate most often consisted of two or more parallel lines with similar electrophoretic localization O antigens (LPS) purified according to *Westphal et al* (41) from *E coli* of other O groups gave similar immunoelectrophoretic patterns with their matching O antisera Consistent with the notion that these precipitates corresponded to O antigen was the observation that a similar precipitate was formed by the S form but not by the R form of an O18 strain This is in agreement with the concept of *Moller* that there is no serological relation between heat stable antigens of R and S forms of *E coli* (28) In this relation it is noteworthy that recent chemical analysis of the O antigen of the O18 strain used in the present work revealed that rhamnose was the only carbohydrate in addition to the basal carbohydrate core presumed to be the same in the R and S forms of this as well as of other coli strains (31)

The purified O6 lipopolysaccharide preparation formed three precipitates with O antisera An additional precipitate only formed with OKH antisera was due to a contamination with K antigen in the O antigen preparation (Fig 2) Whether any other of the three remaining precipitates was formed by an impurity or they were all formed by a heterogeneous O antigen cannot be settled at present Other workers (35) have also obtained more than one precipitate with purified O antigens from *E coli* These observations together with the many cross reactions observed between various *E coli* O groups in agglutination experiments (31) may be taken in support of the suggestion by *Ørskov et al* (31) that the O antigens of *E coli* are complex and composed of several factors as in the *Salmonella* O groups Such a view is consistent with the finding that two precipitinogens were common to the O4 and O18 lipopolysaccharide antigens and one was not (Fig 4) and the similar observation that one of the two precipitinogens in the O7 LPS was also found in an *E coli* O25 strain

The K antigen was identified in the VF antigen preparation from the model strain by i a absorption experiments and because of its high electrophoretic mobility it could be obtained in a pure state by preparative zone electrophoresis Recently K antigens have also been isolated by means of fractionated precipitation with cetavlon which precipitates acidic polysaccharides (5 18 30) The two parallel lines often obtained with the K antigen in immunoelectrophoretic analyses may indicate a heterogeneity of the K antigen Such a heterogeneity

might also explain the finding of the two factors in the model strain but not in another strain which according to the Kauffmann Knipschildt-Vahlne antigenic formula only differs in its K antigen. It cannot be definitely stated however that both of these factors really are K antigens since there may be antigens other than K and not included in the classical antigenic formula which differ in the two strains.

The K2a 2c antigen of the model O6 strain has been classified as a thermolabile L antigen (21). In spite of this we found that it retained its precipitinogenic ability after boiling for two hours. However the K2a 2c antigen native or boiled was precipitated only by OKH antisera but not by O antisera. These properties indicate that it is rather a B antigen. I and F Ørskov have also demonstrated K antigens of the B type in L strains of E coli (29).

E coli bacteria belonging to the O group 6 are often found to be the causative organism in urinary tract infections in children (44). Our earlier studies have shown that children with pyelonephritis caused by E coli form antibodies against the O antigen of the infecting bacteria (3 43). Preliminary experiments however have shown that such children also can form antibodies against antigens other than the O antigen (17). Thus to get a more complete picture of the immune response in patients with urinary tract infections it is necessary to obtain a more detailed knowledge of the antigenic mosaic of the infecting strains.

SUMMARY

The antigenic pattern of an E coli O6 type strain was investigated using immunodiffusion methods. More than 20 antigenic factors could be discerned using various types of antigen preparations. O K and H antigens could be identified. Using O6 O antisera three antigenic factors were found in the purified O6 lipopolysaccharide indicating a possible heterogeneity of the O antigen. Due to its high electrophoretic mobility the K antigen could be isolated by preparative zone electrophoresis.

By comparison of the E coli O antigens of the O groups 1 2 4 6 7 8 18 and 75 using double diffusion technique a cross reaction was observed between O4 and O18 in addition O25 reacted with O7 O antiserum.

A double diffusion method using disks soaked with antiserum was applied for O grouping of E coli strains.

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THE DISTRIBUTION OF TRITIUM-LABELLED BENZYLPENICILLIN IN STAPHYLOCOCCAL ARTHRITIS

An Autoradiographic Study in Golden Hamsters

By

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Received 15 i 69

It is well known that it is often difficult to cure infections in the skeleton and in joints with antibiotics It appears that the substance given does not reach or does not affect the bacteria in certain parts of the focus For this reason a series of investigations about the distribution of antibiotics in infectious lesions in the skeleton and joints have been started As a link in this research programme the distribution of tritium labelled dihydrostreptomycin in experimental tuberculous osteomyelitis and of tritium labelled dihydrostreptomycin and tetracyclin in experimental staphylococcal arthritis have been described in two earlier papers (*Lindberg* 1967 *Lindberg & Lundberg* 1968) This paper in which the distribution of tritium labelled benzylpenicillin in experimental staphylococcal arthritis is described is a continuation on this program

MATERIAL AND METHODS

(A more detailed description of the method is found in the paper of *Lindberg & Lundberg* 1969)

Twenty golden hamsters *weighing about 100* g each were injected in the left knee with 0.05 cc of a suspension of *Staphylococcus aureus* strain Wood 46 containing 10[9] colony forming units per cc This injection produces a standard arthritis described by *Lindberg* (1969) The animals were devided in two groups of ten animals each The first group was injected with *tritium labelled* benzylpenicillin two days and the other group two weeks after the bacterial injection

Each animal received an injection of 0.25 cc of physiological saline containing 3.08 mg of tritium labelled benzylpenicillin potassium per cc corresponding to 0.7 mCi/animal The solution was injected intramuscularly under the left scapula

Two animals of each group were killed 15 minutes 30 minutes 1 hour 3 hours and 6 hours respectively after the injection of the penicillin The infected knee joints were removed *frozen in Hexane* and carbon dioxide snow freeze dried em

This investigation was made possible by a grant from *Alfred Österlunds Stiftelse*

The radioactive penicillin benzylpenicillin T(G) potassium had a specific radioactivity of 0.45 mCi/mMol and was bought from The Radiochemical Center Amersham The radiochemical purity was over 93 per cent



Fig 1

Autoradiogram (top) and corresponding histological section from knee infected two weeks previously The animal was killed 1[illegible] minutes after the injection of tritium labelled benzylpenicillin The large dark area in the autoradiogram ventrally and dorsally in the knee represent abscesses with penicillin in high concentration *The* autoradiogram was exposed 1[illegible] d[illegible] Haematoxylin Magnification × 1°5

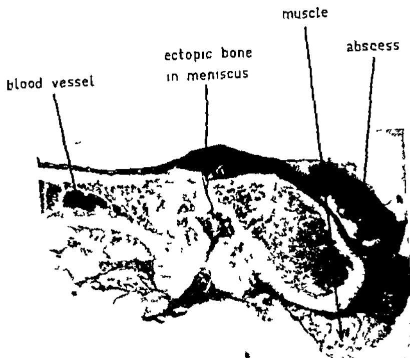


Fig. 2

Autoradiogram of knee infected two days previously. The animal was killed 15 minutes after injection of tritium labelled penicillin. Note the high concentration in the large tibial blood vessel. The autoradiogram was exposed 212 days. Magnification × 12.5.

bedded and tape sectioned. The tape mounted sections were fastened to Ilford G5 autoradiographic plates and the autoradiograms exposed 7–8 months. After exposure plates and sections were processed in the usual way (*Ullberg* & *Lundberg* 1969). The autoradiograms and the corresponding histological sections were afterwards compared by placing the sections over the autoradiograms.

RESULTS

The distribution pattern of the penicillin given two days after injection of the bacterial suspension did not differ with certainty from that found when the penicillin was not given until two weeks after the bacterial injection.

The distribution of the penicillin seen in the infected knee joints of animals at different intervals after the injection are given below.

Already after *15 minutes* the penicillin had diffused into all the tissues of the injected joints except the calcified bone tissue (Figs. 1 and 2). The highest concentration was found in the pus of abscesses and in soft tissues penetrated by inflammatory cells where the blackening of the autoradiograms was of comparable intensity. The blackening over blood vessels was somewhat lower than that over the pus and of

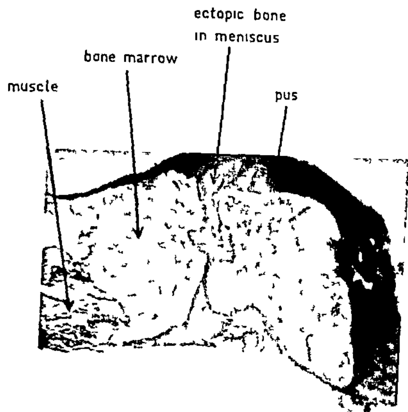


Fig 3

Autoradiogram of knee infected two weeks previously. The animal was killed 30 minutes after injection of tritium labelled benzylpenicillin. The relative concentration of penicillin is the same as that 1 minutes after injection. The autoradiogram was exposed 21[illegible] days. Haematoxylin. Magnification × 1[illegible]5

about the same intensity as that over the periosteum and over the fibrous streaks between the muscle bundles. A still lower concentration was found in joint cartilage, menisci, muscles and bone marrow. The blackening over the epiphyseal lines was very low and resembled that over calcified bone tissue.

After *30 minutes* the distribution pattern seemed to be unchanged (Fig 3).

After *1 hour* the concentration had decreased considerably in all tissues. The concentration in the infected tissues was *still higher than* that in normal tissues (Fig 4).

After *3 hours and 6 hours* the concentration in all tissues was so low that no autoradiograms were obtainable.

DISCUSSION

According to different authors 40–70 per cent of intramuscularly given benzylpenicillin is excreted unchanged in the urine, a small part



Fig 4

Autoradiogram (top) and corresponding section from knee infected two weeks previously The animal was killed one hour after injection of tritium labelled penicillin The concentration of the penicillin is still somewhat higher in the pus in the knee joint than in other soft tissues Note that the autoradiogram has been exposed 2,3 days to obtain this degree of blackening For this reason it is not possible to compare the absolute blackening of this figure with that of Figs 1–3 Haematoxylin Magnification × 12 5

is also excreted with the bile whereas the remaining part 30 50 per cent is excreted in the urine as inactive metabolites (*Ullberg* 1954 *Walter & Heilmeyer* 1965) *It is difficult* to obtain more exact values as these are varying with the different methods and animals used *Considerable individual* variations of the values are also found when the *excretion in human beings* is investigated

In this investigation it is thus *possible that parts of the blackening* of the autoradiograms is caused by radioactive *metabolites* As the definitive distribution pattern however is reached already 15 minutes after the injection of the penicillin this risk is very small as no large amount of the penicillin can have been broken down in such a short time (*Ullberg* 1954) The risk that the breakdown of the *penicillin occurs extremely rapid in e g* pus or inflammatory cells naturally can exist and *must be borne* in mind In spite of this the method has certain advantages over *in vitro* determinations of the penicillin concentration in concentration in biopsy specimens as *it is possible to assess the relative* concentrations of the penicillin in the various tissues at a *microscopical level* without contaminating them with blood or other tissue fluids containing different amounts of the antibiotic

Ullberg (1954) using autoradiographic technique has investigated the penetration of ^{35}S labelled benzylpenicillin into experimental abscesses produced by intramuscular and subcutaneous injections of *Corynebacterium pyogenes* in mice The abscesses were between 6 and 30 days old The penicillin was found to penetrate the abscess wall and was 30 minutes after the injection found in a thin layer of the abscess content immediately inside the wall The concentration of the penicillin in this layer was higher than that in the abscess wall and was higher than or comparable to that in the blood but most part of the abscess content was totally devoid of penicillin

In this investigation the penicillin had completely penetrated the content of all abscesses and all other infected tissues already 15 minutes *after the injection* Moreover a higher concentration was found in these tissues than in the blood and in other tissues as long as autoradiograms could be obtained

As to the penetration of benzylpenicillin into the abscesses the difference in the results obtained by *Ullberg* and the results of this investigation can be caused by several factors as *e g* differences in the age the content or the wall of the abscesses However the results *do not allow* any *conclusions about* this question

The results from both investigations however show that benzylpenicillin has a tendency to be concentrated in pus The reason for this is not known As the concentration in the pus was higher than that in the blood or any other part of the histological section it seems reasonable to assume that the penicillin (or *metabolites of the penicillin*) in some way was bound to the constituents of the pus *Diffusion alone* cannot explain this *increase of concentration* from blood to pus The

same tendency was found for dihydrostreptomycin both in pus from staphylococcal arthritis and in tuberculous pus (*Lindberg* 1967 *Lindberg & Lundberg* 1969) where the high concentration was shown to depend on a reversible binding of the dihydrostreptomycin to cellular remnants stainable with haematoxylin probably nuclear remnants

SUMMARY

The distribution of benzylpenicillin in experimental staphylococcal arthritis has been studied with autoradiographic technique After intramuscular injection the penicillin is found to penetrate into the infected tissues even abscesses It is also found to be concentrated in pus compared to normal soft tissues and blood The reason for this concentration is discussed

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BRIEF REPORT

PEROPERATIVE EVALUATION OF THE JUXTAGLOMERULAR APPARATUS IN HUMAN KIDNEYS

A Rapid Frozen Section Technique Especially Applicable to Hypertensive Patients

By *Poul Faarup* and *Michael Petri*

In human hypertension structural changes in the juxtaglomerular apparatus (JGA) of the stenosed kidney have been found to be of diagnostic interest (e g *Genest et al* 1966 *Faarup et al* 1967 1969)

In a number of cases it may be undesirable to run the risk of the pre operative needle biopsy Thus a peroperative histological method by which the occurrence of hyperplasia or hypergranulation of the epithelioid cells in the JGA could be rapidly estimated in a peroperative biopsy has been considered clinically important. In cases with segmental lesions biopsies may aid the surgeons in localizing the involved segment during the surgical procedure

Crocker (1964) has shown that the use of general tissue stains on frozen sections may demonstrate cases *of pronounced hyperplasia of the JGA In such preparations* however the identification of the juxtaglomerular cell types is difficult and the *juxtaglomerular granules will remain unstained Therefore a staining method* which gives a reliable definition of these structures has been worked out

Technique

Cryo technique Unfixed kidney tissue was frozen in a mixture of isopentane and dry ice (app *—65 C) or in isopentane cooled by liquid nitrogen* (app —160 C) Thin sections were cut on a freezing microtome or—preferably—on a cryostat

Staining procedure A number of quantitative variations of the periodic acid Schiff method was tried in order to speed up the procedure It was found that a *reliable and reproducible method for frozen sections was obtained by halving* the time traditionally used while keeping the fluids in constant agitation by a magnetic stirrer

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Figs 1–4

Fig 1 In the wall of the afferent arteriole (AA) several epithelioid cells with *juxtaglomerular granules* may be seen (arrows) MD Macula densa EA Efferent arteriole (C Cell group of Goormaghtigh (Frozen section PAS × 490)

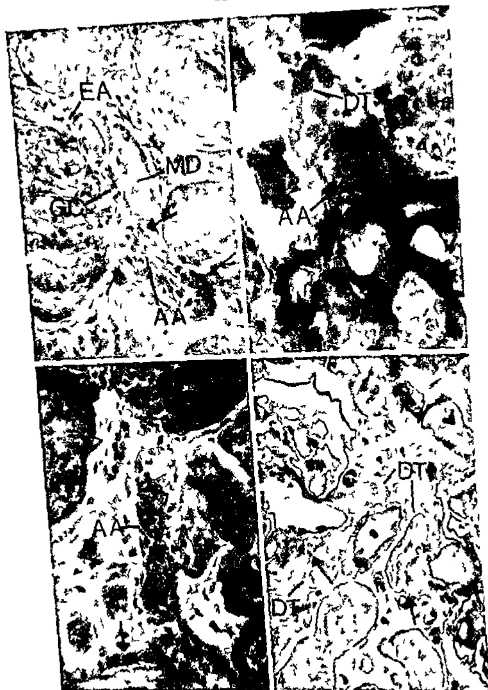
Fig 2 Tangential section of an afferent arteriole (AA) in which juxtaglomerular granules are easily *identified in the* cell DT Distal tubule (*Frozen section* PAS × 710)

Fig 3 Juxtaglomerular *granules are found in most epitheliod* cells of the afferent arteriole (AA) Epithelioid cells occur in the pre *afferent arteriole as* well (arrow) Moderate *interstitial fibrosis is present* (*Frozen section* PAS × 560)

Fig 4 Granulated epithelioid cell (arrows) *in the* juxtaglomerular appartus close to the distal tubule (DT) The glomerular and tubular basement membranes are well defined (Paraffin section PAS × *460)*

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EA
MD
GC
AA
DT
AA
AA
DT
DT

Schiff's reagent was prepared according to the "cold" Schiff *procedure of Lillie* (1965). Counterstaining was done by *celestine blue picric acid after Petri* (1968). The contrasting green colour obtained greatly facilitates the recognition of PAS positive structures such as juxtaglomerular granules.

Sulphite rinses (0.5 per cent) were prepared fresh from a 10 per cent stoc solution of sodium metabisulphite.

		Time
1	Periodic acid 1 per cent	5 min
2	Rinse in tap water	2 min
3	Schiff's reagent	6 min
4	Sulphite rinse 0.5 per cent	3 × min
5	Rinse in tap water	min
6	Celestine blue	2 min
7	Saturated aqueous picric acid	2 min
8	Dehydrate quickly by absolute ethanol clear mount in DPX	1 min
	Time of staining procedure	25 min

Results

While the 25 minute frozen section procedure is being carried out one may of course do a general tissue stain of short duration on a few sections. We found van Gieson's stain well suited for estimating general kidney morphology. However the PAS method gives a differentiation of the general kidney structure which is superior to general tissue stains used for routine diagnostic work. Thus the different tubular segments of the nephron as well as glomerular and arterial morphology are well demonstrated. In Fig 1 a JGA from the stenosed kidney of a patient with renovascular hypertension is seen. In the distal part of the afferent arteriole and in the Goormaghtigh cell region a large number of epithelioid cells can be identified. In some of these cells the juxtaglomerular granules are recognized. In thin sections it is possible to demonstrate single granules in the cytoplasm (Fig 2).

The results of the present method applied to surgical cases of hypertension will be published separately (*Faarup et al* 1969).

In connection with the present studies on a method for rapid evaluation of the juxtaglomerular apparatus in peroperative biopsies we investigated the value of the modification of the PAS method previously described by *Petri* (1968) for *Helly fixed paraffin embedded tissue*. A definition of histological details in the JGA was better (Fig 4) than that obtained with the Bowie stain. The latter is a somewhat more tricky and less reproducible method and even more time consuming. Besides with the PAS method the different cell groups are well distinguished, cellborders are more easily seen and the juxtaglomerular granules stand out in a bright red colour. It should be mentioned however that in some species of animals e.g. in rats the PAS method for the JGA is inferior to Bowie's method.

Summary and Conclusions

By the frozen section PAS method described a rapid clinically useful staining method has been devised for peroperative kidney biopsies particularly well suited for estimating alterations in the juxtaglomerular apparatus. The method was found suitable also for paraffin embedded tissue preferably fixed by *Helly's fluid*. In addition in both frozen and fixed tissue general kidney morphology is well differentiated including important diagnostic details e.g. the glomerular basement membrane.

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Acta path microbiol scand. 76 329-336 1969

TRANSACTIONS OF THE MEDICAL MICROBIOLOGY DIVISION OF THE SWEDISH MEDICAL SOCIETY

Annual Meeting 28th November-1st December 1968

IMMUNOLOGY

Vera Beckman & *G Möller* Transplantationlaboratory The Seraphimerhospital Stockholm SUPPRESSION OF LYMPHOCYTEMEDIATED CYTOTOXICITY *IN VITRO* BY ANTISERA AGAINST HUMAN GAMMAGLOBULINS

In our experiments rabbit anti era against different human γ globulin classes (IgG IgA and IgM) were used With each of these antisera it was possible to demonstrate a weak stimulation of the DNA s nthesis in vitro in human lympho cytes from peripheral blood in 2 expe im nts out of 7 perf rmed The maximum of the stimulation was reached on the fifth day In the other experiments these anti sera ha l no demonstrable effect on the DNA synthesis Furthermore studies of the cytotoxic effect of human lymphocytes against human fibroblasts in vitro were per formed The lymphocyte were added to monola ers of fibroblasts on pre indicated sites where the fibroblasts get damaged and give a clear plaque which is graded from + to ++++ Normal lymphocytes were induced by phytohaemagglutinin (PHA) to kill allo enic fibroblasts and this cytotoxic effect could be partly or totally suppressed by each of these antisera In the same dosages these antisera alone with out the presence of PHA could not be shown to induce any cyt toxicity in the l mphocytes against fibroblasts

B Bloth & *S E Svehag* Department of Immunology National Bacteriological Labo ratory Stockholm ULTRASTRUCTURE OF PAPAIN DIGESTED IgM FRAGMENTS

To be publi hed in J Exp Medicine 1969

Ö Strannegård The Department of Virology Institute of Micr biology University of Gothenburg REAGIN RESPONSE IN RABBITS FOLLOWING PARENTERAL AND ORAL ANTIGEN ADMINISTRATION

F rmation of reaginic (h moc totropic) antibodies was obtained in rabbits follo ving immunization with bovine serum albumin (BSA) or dinitrophenylated BSA (DNP BSA) By means of chromatographic experiments and absorptions with specific anti heavy chain an l anti light chain sera strong evidence was obtained that the rabbit reagins bel ng in an immunoglobulin class which is distinct from IgG IgA and IgM

The initial logarithmic phase of the primary reagin response proceeded rapidly indicating a doubling time of 7 8 hours The anti DNP reagins generally appeared earlier and disappeared more rapidly from the circulation than did the anti BSA reagins After booster antigen inje tions a secondary type of reagin response was evoked in some cases This response was more readily obtained in rabbits with low

Acta path microbiol scand
76 329 336 1969

serum titres of agglutinating antibodies than in those with high titres suggesting the possible existence of antibody induced suppression of reagin response

Following oral administration of antigen a reagin response was evoked in one out of 13 rabbits which had been fed BSA and in one out of 13 rabbits fed egg albumin The serum titres of anti BSA reagins persisted for several months following cessation of antigen administration

Hederstedt B Lind J & Lundblad C National Bacteriological Laboratory Stockholm STUDIES ON HEAT AGGREGATION OF SERUM PROTEINS

Normal human sera were heated up to 40 C–63 C for varying time intervals Sephadex G–200 gel filtration of heated sera revealed a protein rise of the 19S peak and a decrease of the 7S and albumin peaks In the case of a serum heated at 63 for 10 minutes at 56 for 30 minutes or at 40 C for 5 days the 19S peak increased by 140 80 and 20 per cent respectively

With gel diffusion precipitation lines were formed between material from the increased 19S peaks and anti IgG and anti albumin sera Using a 390 cm column of Bio Gel A–15 m the macromolecular material was separated further and heat aggregation of IgM and IgA was demonstrated

Diluted syphilis sera of high TPI and FTA antibody titres (IgG only) were heated at 56 C for 24 hours Sixty five per cent of the 7S globulins were aggregated which led to a decrease of more than 2 two fold TPI titre steps but only one FTA titre step Neither TPI and FTA antibodies nor anti complementary activity was demonstrated in the enlarged 19S peaks The following questions were raised May antibodies be heat inactivated without being aggregated? Are certain IgG antibodies more readily aggregated than others?

VIROLOGY AND CELL BIOLOGY

Marianne Forsgren Central Bacteriological Laboratory of Stockholm City
ECHOVIRUS 6 ANTIGENS

The antigen composition of echovirus 6 preparations has been studied with immunodiffusion immunoelectrophoresis staining of precipitates with the nucleic acid staining acridine orange antigen separation in CsCl density gradient complement fixation and electronmicroscopy Precipitation against human convalescent sera and acridine orange staining of the precipitates show that native preparations of echovirus 6 contain two different antigens one heat labile RNA staining antigen (N) and one heat stable RNA negative (H) antigen In immuno electrophoresis at pH 8 2 (agarose) the two antigens migrate at equal velocity at pH 7 0 the N antigen migrates slower than the H antigen In CsCl density gradient a separation of the two antigens is achieved one band (density 1 [illegible] g/cm³) carries max infectivity complement fixing and precipitating activity of N character The other band (density 1 30) carries low infectivity complement fixing and precipitating activity of H character Electron microscopy (carried out by Bjorn Bloth Immunol Dep SPL) shows complete virions in the N band and empty capsids in the H band

Antibodies against both the N and H antigens are developed during echovirus 6 infections in man

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This study was supported by grants from the WHO

Acta path micr biol scand
76 329 336 1969

J Anderst & H O Sjögren Department of Medical Microbiology University of Lund Lund Sweden CROSS REACTION BETWEEN TUMOUR ANTIGENS OF ADENO 7 AND 12 SARCOMAS

With a Cr 51 cytotoxic test developed to work on nonlymphoid target cells hamster adeno 7 and 12 tumours were shown to be sensitive to the tumour specific cytotoxic antibodies of mouse antisera against syngeneic adeno 12 tumours When the same sera were tested against control hamster cells (BHK-C13) no antibody activity could be detected the isotope release being similar with active and with inactive complement The cytotoxic effect on hamster adeno 7 tumour cells was confirmed by the colony inhibition technique Furthermore it was demonstrated by transplantation tests in mice that the hamster adeno 7 tumour cells were immunogenic causing an isograft immunity to adeno 12 tumours similar to that induced by adeno 12 hamster and mouse tumour cells

G Wadell Karolinska Institutet Stockholm HAEMAGGLUTINATION WITH ADENOVIRUSES BELONGING TO ROSEN S SUBGROUPS II AND III

These adenoviruses have previously been shown to agglutinate rat red cells completely and partially respectively The haemagglutination with purified virions and crude virus preparations enhanced by heterologous antisera of serotypes 1 2 4 5 6 (III) and 9 15 (II) was studied With all these preparations complete agglutination of rat red cells and also haemagglutination of human O was demonstrated

Experiments concerning adsorptions of virions by red cells competitive interaction of incomplete haemagglutinins (HA s) (i e pentons and fibres) with the agglutination by virion associated HA and the effect of receptor destroying enzyme on red cells were performed The results suggest that haemagglutination patterns were dependent of the relation between incomplete and complete HA s the number of receptors on the red cells and possibly also qualitative differences between the receptors

Haemagglutination of rat cells at 20 C by the tested serotypes was considered more efficient than the use of human O red cells since agglutination of the latter was more sensitive to the presence of incomplete HA s The former system was used to determine the optimal antigen preparations of serotypes belonging in subgroup III for haemagglutination inhibition tests Unfractionated virus preparations containing heterologous antisera were recommended

Pettersson Ulf & Höglund S Department of Microbiology The Wallenberg Laboratory and The Institute of Biochemistry Uppsala PURIFICATION AND CHARACTERIZATION OF PENTON ANTIGEN FROM ADENOVIRUS TYPE 2

The penton antigen from adenovirus type 2 has been purified by freon extraction high speed centrifugation exclusion chromatography on 6 per cent agarose and preparative polyacrylamide electrophoresis

The pure product sediments as a homogeneous peak with an S value of 10 7 ± 0 3 and analytical polyacrylamide electrophoresis and immuno electrophoresis reveal one single component

Immunodiffusion shows that the vertex capsomer is mainly subgroup specific while the fibre contains two specificities one subgroup and one type specific

The pure penton acts as an indirect haemagglutinin in the presence of heterotypic penton antibodies but is in the order of 5 times less effective than the fibre on a weight basis as could be expected from the difference in molecular weight

Pure penton induces cytopathic effect at a level of 0 1 μg penton/10⁶ KB cells This effect could be blocked by antisera against pentons but not by antisera against fibres

Trypsin abolishes the cytopathic effect of the penton completely but leaves part of the antigenicity of the vertex capsomer intact

Pure penton induces high titres of neutralizing antibodies when measured by the fluorescent focus assay but not when measured by the plaque assay

Amino acid analysis shows that the primary structure of the vertex capsomer must be different from that of the hexon provided both structures are composed of one single polypeptide chain

Electronmicroscopy reveals the classical structure composed of a spherical part with a central hole—the vertex capsomer—and the elongated fibre

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BACTERIOLOGY

H O Hallander Kathrine Dornbusch & G Laurell Department of Medical Microbiology University of Uppsala Uppsala Sweden DETERMINATION OF METHICILLIN RESISTANCE INFLUENCE OF STAPHYLOCOCCAL HETEROGENEITY ON THE DISC DIFFUSION METHOD

In this study 48 methicillin resistant penicillin β lactamas producing strains of *Staphylococcus aureus* (met p) selected according to *Barber & Waterworth* (1) were compared to 50 methicillin sensitive but still penicillin β lactamas producing strains (met p) All met p strains were shown to be heterogeneous with a very constant frequency of about 1 cell per 10⁶ resistant to 400 μ and 1 cell per 10⁴ resistant to 12 5 μ methicillin Colonies selected from plates with 400 μ methicillin quickly resegregated when cloned on ordinary medium

In a strictly standardized disc (30 μ) diffusion test with ordinary sheep blood agar medium and incubation at 37 C according to *Ericsson et al* (2) 40 per cent of the resistant strains were falsely negative The zone of inhibition varied from 0 to 25 mm None of the met p variants gave a zone less than 26 mm

Because of the very constant heterogeneity this variation must be due to factors in the disc diffusion method unfavourably affecting the relatively instable methicillin resistant mutants

A somewhat better separation between the two groups of strains was demonstrated with weaker discs (10 μ)

When the medium was osmotically stabilized with 5 per cent NaCl the zones for met p bacteria were on an average 4 5 mm less with no falsely negative results No change at all could be shown for met p strains

The best results were obtained when the strains were incubated on *ordinary sheep* blood agar medium at 30 Now the inhibition zone were on an average 19 5 mm less for met p with no change for met p variants

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Carl Ericson Institute of Clinical Bacteriology Allmanna sjukhuset, Malmo

RESISTANCE TO ACRIFLAVINE AND CADMIUM AND CHANGED PHAGE REACTIONS – MARKERS OF A NEW STAPHYLOCOCCAL PENICILLINASE PLASMID?

Staphylococcus aureus strain Fr 837–2 produced penicillinase (*pen*) and was resistant to several antibiotics It was resistant to acriflavine (MIC 200 μg/ml) and Cd (MIC 8 0 $\times$ 10^{-5} M) but sensitive to Hg (MIC 2 3 $\times$ 10^{-5} M) It was non typable (NT) with standard phages at 1000 $\times$ RTD

Penicillinase negative (*pen*) variants were isolated on Water blue agar (0 01 per cent water blue Merck Darmstadt) containing 0 05 μg/ml methicillin After 2 days at 37 C and flooding with 3 per cent benzyl penicillin *pen* colonies turned dark blue

Pen clones were sensitive to acriflavine (MIC 12 5 μg/ml) Cd (MIC 2 4 $\times$ 10^{-6} M) and Hg^{2} (MIC 2 3 $\times$ 10^{-6} M) and had the phage type 7/47/54/75 at RTD but otherwise seemed identical with the parent strain No difference in lysogenicity could be shown

All markers *i e* penicillinase production the NT state resistance to acriflavine and cadmium were segregated together and at increased frequencies on treatment with acridine orange (25 μg/ml) or incubation at 42 C

The change of phage reactions was from inhibition reactions at 1000–10 000 $\times$ RTD to plaques at RTD There may be some analogy between this effect and loss of plasmid induced phage restriction in *Enterobacteriaceae*

As yet the data point to acriflavine resistance and changed phage reactions as probable markers previously not described of a penicillinase plasmid

Transduction experiments are under way

Paula B anefors & Pål Henry Jeppson Department of Bacteriology and the Department of Ear Nose and Throat Diseases Sahlgrenska Sjukhuset Goteborg

HAEMOPHILUS INFLUENZAE AND ACUTE EPIGLOTTITIS – BACTERIOLOGICAL AND SEROLOGICAL STUDY

Six patients four children and two adults with the clinical diagnosis of acute epiglottitis were studied from bacteriological and serological points of view

Haemophilus influenzae type b was isolated from the epiglottic region in all the patients Blood cultivation before antibiotic treatment showed *H influenzae* type b in five cases

Blood samples for the serological analyses were taken on admission to hospital after one week and after three weeks The serological analyses were performed with the double diffusion in gel technique Free capsular substance b was demonstrated in the first serum sample of three patients and was considered as a sign of massive bacteraemia Capsular b antibodies could not be demonstrated in the first serum sample from any patient and only irregularly in the second and third samples All patient however had precipitating antibodies against the cell wall antigen (O antigen) even in the first serum sample

It is therefore possible that a local allergic reaction superimposed on the inflammatory reaction caused by the bacterial invasion results in the often excessive swelling of the epiglottic tissue

Acta path microbiol scand
76 329 336 1969

Inna Stina Malmborg S O Eriedahl B Nystrom Sig Serm & I Sjöberg Surgical Clinic and Laboratory for Clinical Bacteriology Karolinska Hospital and the National Bacteriological Laboratory Stockholm INFECTIONS WITH PSEUDOMONAS IN A BURNS UNIT

The contamination of burns and of the ward environment with *Pseudomonas aeruginosa* was studied in a burns unit for 6 months

The study comprised 41 patients *Pseudomonas aeruginosa* strains were isolated from 17 of the patients mainly from patients with extensive burns Only a few of these patients had clinically observable infections This is probably mainly due to the principles of treatment open treatment with warm dry air and restrictive antibiotic therapy *Pseudomonas aeruginosa* strains were also isolated from the ward environment mainly from wash basins and sinks

Fifty three phage patterns were found among 255 pseudomonas strains Five phage patterns of similar type were grouped together These 5 patterns comprise 47 per cent of the isolated strains

An attempt was made to control the contamination of the environment by routine use of Sanivit a detergent for cleaning wash basins and sinks The detergent is bactericidal due to release of chlorine The introduction of this hygienic procedure was followed by a temporary reduction in the number of strains isolated from the environment The number of strains isolated from the patients was considerably reduced

H Cnarpe & I Olding The Institute of Medical Microbiology Department of Bacteriology and The Institute of Pathology University of Uppsala Uppsala
RETROGRADE PROTEUS PYELONEPHRITIS IN RABBITS

In Proteus induced urinary tract infections the urinary findings of leucocytes are often sparse and irregular Earlier in vitro experiments have shown that leucocytes are rapidly disintegrated in alkaline media i e when Proteus organisms grow in urine

This work was done to test these findings in experimental pyelonephritis Rabbits were given retrograde pyelonephritis with a method modified from Frat The urinary findings of leucocytes bacteria and the urinary pH were studied After sacrifice urine and kidney cultures were made and the microscopic picture of the inflammatory changes in the kidneys were studied

In 75 per cent of the rabbits infected with Proteus there was a tendency to urinary pH levels above 8 0 The urinary findings of leucocytes in those cases were sparse in spite of pronounced inflammatory reaction in the kidneys In 25 per cent of the Proteus infected cases there was no rise in the urinary pH and large numbers of leucocytes were found in the urine

With the aid of fluorescent antibody technique the bacteria were found to be localized interstitially in the kidneys

J Holmgren G Eggertsen Lars Åke Hanson & K Lincoln Institute of Medical Microbiology University of Goteborg Goteborg Sweden STUDIES ON THE K AND H ANTIGENS IN AN E COLI O6 TYPE STRAIN

An *E coli* strain serotype O6 K?a ?c H1 was studied by immunodiffusion methods with the aim of identifying the K?a c and the H1 antigens From previous investigations this strain used as med I strain, was known to contain more than 0 antigenic factors three of which were related to the O6 antigen

Acta path micr biol scand
76 329 336 1969

By comparison in double diffusion of the model strain and an O? K13 H1 strain and by absorption of anti O? K?a ?c H1 immune serum with the last mentioned strain the K?a ?c antigen of the model strain was identified In immuno electrophoretic analysis the K2a ?c antigen was found to be the fastest migrating antigenic factor in the model strain This made it possible to isolate it by means of preparative zone electrophoresis in Sephadex G-?5 The isolated K antigen could sensitize untreated sheep red blood cells for passive haemagglutination by K?a ?c antibodies This capacity as well as its precipitinogenic ability was practically unaffected by boiling for 2 hours although the model strain is classified as a thermo labile L antigen strain The antibody response in rabbits against the K?a 2c antigen was studied *inter alia* by passive haemagglutination A rapid response with passive haemagglutination titres up to 1/5000 was observed after a single i v injection of *E coli* O6 K?a ?c H1 bacteria

Many of the antigenic factors in the model strain were also found in *E coli* O? K1 H4 and O14 K? H By absorption of anti O6 K2a ?c H1 with bacterial extracts of these strains and with the heat stable antigen factors of the model strain an anti serum was prepared which was monospecific for an antigen presumably the H1 antigen which by comparative double diffusion was identified in the complex antigenic pattern of the model strain

Karl Axel Karlsson S Dahlstrand E Hanko & O Soderlind Research Institute of National Defence Sundbyberg National Bacteriological Laboratory and National Veterinary Institute Stockholm ON THE USEFULNESS OF THE FA TECHNIQUE IN THE DIAGNOSIS OF TULARAEMIA

In connection with an epidemic outbreak of tularaemia in 1967 ?99 sylvatic animals predominantly hares have been investigated regarding tularaemia In all cases the FA technique was applied besides histopathological and/or conventional bacteriological investigations

In 124 cases all three methods were used Complete agreement was obtained in 85 cases (30 positive 55 negative) In 34 cases the results of the histopathological and FA investigation were positive whereas the bacteriological investigations were negative 4 cases were positive with the FA technique but could not be confirmed with the other methods

In one case the FA technique gave a negative result but the other two diagnostic procedures were positive

In 10 animals the FA technique was compared with histopathological procedures only The results were in agreement in 103 cases (57 positive 46 negative) The remaining case were positive histopathologically but negative with FA technique

In 146 guinea pigs which had been inoculated with material from cases suspected of tularaemia infection the results of cultural and FA procedures were compared Both methods gave the same diagnosis in 136 cases In 9 cases the FA technique gave a positive result whereas the cultivation was negative In one case the FA test was negative whereas the cultivation method was positive

In experimentally infected rabbits tularaemia bacteria could be detected in the ? even after storage at room temperature for 2? days post mortem

According to this study the results of the FA technique for the detection of tularaemia in field material are in good agreement with the results of the time consuming histopathological examination and superior to the conventional bacterio logical investigation

Acta path microbiol scand
76 329 336 1969

F Hammarström & *Klara Thiringer* Department of Clinical Bacteriology The Sundswall Hospital Sundsvall THE INFLUENCE OF TEMPERATURE ON THE TRANSPORT OF MENINGOCOCCAL, GONOCOCCAL AND URINE SPECIMENS

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DEGENERATIVE CHANGES IN THE PROXIMAL RENAL TUBULES FOLLOWING ADMINISTRATION OF BACITRACIN

An Ultrastructural Study in the Mouse

By

ASMUND KJÆRHEIM and ODD E HANSSEN

Received 23 xii 68

Plasma proteins are normally filtered through the glomeruli of the kidneys and are subsequently reabsorbed by the epithelial cells of the proximal tubules Accordingly proteinuria can be caused by three different mechanisms First protein may be excreted in the urine if the quantity of filtered protein surpasses the reabsorptive capacity of the tubular cells This may occur if the concentration of normal or pathological proteins in the blood plasma is increased or secondly if increased amounts of plasma proteins are filtered because of abnormal permeability of the glomeruli

The first of these mechanisms has been demonstrated experimentally by way of intravascular infusion of different proteins Among others *Ericsson* (3) and *Miller* (14) used homologous haemoglobin and studied the tubular reabsorption of this protein with the electron microscope Proteinuria due to the second mechanism i e increased permeability of the glomeruli occurs in clinical nephrosis in man and in nephrosis induced by aminonucleoside in experimental animals In these conditions the most striking change within the glomeruli is fusion of the epithelial foot processes Using ferritin as a tracer it was demonstrated ultrastructurally that the permeability of the glomeruli is increased in aminonucleoside nephrosis (4) These authors claimed that the primary lesion is within the basement membrane and that fusion of foot processes occurs secondarily

In both types of proteinuria vacuoles and round dense aggregates are observed electron microscopically in the tubular cells Most of these dense structures are thought to represent reabsorbed protein and probably correspond to the hyaline droplets observed by light microscopy in different types of proteinuria

Thirdly proteinuria will follow when the reabsorptive capacity of the tubular epithelium is decreased However there are few if any

clear cut examples of this category in which the glomerular function is undoubtedly normal In the present paper which is the first comprehensive report on the fine structural lesions within the proximal tubules following bacitracin we suggest bacitracin intoxication as an experimental model of this type This suggestion is based on the observations made in this study and is supported by previous light microscopical demonstrations of well maintained glomerular filtration (7) and reduced tubular reabsorption of Evans blue (6) in this condition

MATERIALS AND METHODS

Twenty one female white mice in groups of 7 were given a single intraperitoneal injection of 15 000 I U of bacitracin (A/S Apothekernes Laboratorium for Special preparater Oslo) per kg body weight The animals were killed with ether after 3 6 and 18 hrs Thin slices of kidney tissue were immediately immersed in chilled 1 per cent osmium tetroxide in veronal acetate buffer at pH 7 4 for 2 hrs dehydrated in acetone and embedded in Vestopal W Alternating thick sections for light microscopical orientation and ultrathin sections for electron microscopy were cut on an LKB Ultrotome I The sections for light microscopy were stained with a solution of 0 1 per cent toluidine blue in phosphate buffer at pH 9 0 for 2–3 min the ultrathin sections were stained with lead according to Karnovsky s method B (12) A Siemens Elmiskop I electron microscope equipped with 50 microns platinum objective apertures and double condensor illumination was used for the electron microscopy

RESULTS

Light Microscopy

The major portion of the lumens of the proximal tubules were patent and degenerative changes in the form of dark droplets or bodies were observed within the tubular epithelium Protein casts were found in the lumen particularly in the distal and collecting tubules The glomeruli were essentially normal

The pathological lesions were present after all time intervals studied but were more pronounced in the animals sacrificed 18 hr after the administration of bacitracin As the lesions were qualitatively identical the results are taken together in the following

Electron Microscopy

The fine structure of the glomeruli did not deviate from the normal as illustrated in the survey electron micrograph (Fig 1) The endothelial cells possessed fenestrations in normal amounts (Fig 2) The basement membrane was not thickened and the spaces between the foot processes were within the normal range of 200–700 Å (18)

The main pathological changes occurred within the proximal tubular cells and were observed in (1) the apical portion of the cytoplasm (2) the dense bodies (3) the endoplasmic reticulum and (4) the mitochondria



Figs 1–2

Fig 1 Survey picture of several cells from the renal glomerulus of a mouse 18 hr after a single injection of bacitracin No pathological changes are visible × 7 000

Fig 2 Portion of glomerulus from bacitracin treated mouse (18 hr) The slit pores between the epithelial foot processes are of normal size There is no thickening of the basement membrane Normal number of fenestrations in the endothelial cells × 15 000

(1) *The apical cytoplasm* This region was narrower than normal and contained vesicles of varying sizes (Fig 3) Vacuoles containing a somewhat denser material as seen in normal tubular epithelium were not observed after administration of bacitracin The microvilli of the brush border were less closely packed than normal (Fig 4)

(2) *Dense bodies* One of the most prominent changes in bacitracin intoxication was the presence of numerous membrane bounded dense bodies with heterogeneous contents (Figs 3 4 5 and 9) The majority of these bodies were larger than the dense bodies usually observed in other cells and measured 0 5–1 micron They contained parallel stacks of electron dense lamellae a dark homogeneous material resembling lipid and finely granular masses of varying density In some places remnants of cytoplasmic structures such as mitochondria and ribosomes were observed within the dense bodies The large dense bodies were observed throughout the cell and were not as in normal cells restricted to the apical area

(3) *Agranular endoplasmic reticulum* This organelle was observed as clusters of closely packed smooth vesicular and tubular elements measuring 200–700 Å in diameter (Fig 6) In other areas of the cytoplasm membrane pairs were observed surrounding mitochondria (Fig 7) or round homogeneous structures resembling microbodies (Fig 6) The paired membranes formed spirals which were often continuous with small tubules (diameter 200 300 Å) lying at the inner or the outer surfaces of the spirals (Fig 7)

(4) *Mitochondria* In most blocks the mitochondria were adequately fixed *i e* they were not swollen and their inner membranes were well preserved (Figs 8 and 9) Several mitochondria contained dense intramatrical granules 500–800 Å in diameter *i e* larger than those occurring in normal mitochondria of the tubular cells The outline of the granules was irregular sometimes polycyclic with a central less electron dense area

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Figs 3–4

Fig 3 Tubular epithelium 18 hr after administration of bacitracin *The apical* portion of the cytoplasm is narrower than in untreated animals A large dense body (Db) with heterogeneous membrane bound d contents i visible within the narrow tubular cell × [illegible]1 000

Fig 4 Tubular epithelial cells following treatment with bacitracin for 18 hr The lumen is patent The microvilli are irregular and some of them have been detached Numerous dense bodies (Db) are visible throughout the cytoplasm × 16 000


Db
3

DISCUSSION

The observation of normal glomeruli in bacitracin treated mice is in good agreement with previous morphological and physiological data to the effect that the glomerular filtration is well maintained after administration of bacitracin (6 7)

The observation of patent tubular lumens in bacitracin intoxication sheds some light on the problem of agonal and early postmortal processes leading to changes in the tubular lumen In normally functioning kidneys the lumen is considered to be patent (3 8) In kidneys fixed by immersion however the tubular lumens are almost invariably collapsed The reason for this seems to be that the tubular urine is reabsorbed post mortem On the other hand the tubular reabsorption of urine and subsequent collapse of the lumen can be prevented by mannitol due to the osmotic activity of this substance (8) Similarly the presence of patent lumens in bacitracin intoxication may be caused by increased osmotic activity of proteins in the tubular urine since the reabsorption of proteins is decreased in this condition

The narrow apical zone containing numerous vesicles of varying size has the same appearance as in kidneys fixed *in vivo* (9) This zone also has some features in common with the appearance after osmotic diuresis induced by mannitol Here again the increased osmotic activity of the protein rich tubular urine in bacitracin intoxication induces a picture resembling the *in vivo* situation By contrast normal tubules fixed by immersion fixation exhibit a considerably broader apical zone with vacuoles containing dense material (14)

The presence of large amounts of dense membrane bounded structures resembling digestive vacuoles and autophagic vacuoles was a typical finding in the majority of proximal tubular cells in this study *Ericsson* (3) and *Miller* (14) investigated the ultrastructural changes in the tubular cells following glomerular filtration of intravascularly injected haemoglobin They followed the tubular absorption of haemoglobin and its transformation within vacuoles to structures morphologically identical with those observed in this study Since the reabsorption of protein was decreased in bacitracin intoxication (6) the material within the membrane bounded dense bodies probably origi

Figs 5-6

Fig 5 Dense body within a tubular epithelial cell from a bacitracin treated mouse. The body consists of myelin figures a dense homogeneous substance resembling lipid (L) and finely granular masses × 50 000

Fig 6 Accumulation of agranular endoplasmic reticulum (Er) in a tubular epithelial cell following treatment with bacitracin for 18 hr Numerous tubules and vesicles 200 300 Å in diameter are present within a rather narrow area To the right two membrane limited structures consisting of a relatively electron dense finely granular substance are visible (microbodies Mb) × 30 000

nates from the cytoplasm rather than from the tubular lumen The major portion of large dense bodies probably reflects cell degeneration caused by the toxic influence of bacitracin and they should therefore be considered to be autophagic vacuoles However the possibility remains that at least some of the dense bodies may contain bacitracin taken up by the cell either from the blood stream or from the tubular lumen during the initial phase of intoxication thus being digestive vacuoles (1 2)

The lesions observed in the endoplasmic reticulum of the tubular epithelial cells were almost identical with the smooth tubules and lamellar spirals seen in hepatocytes after treatment with α naphtol isothiocyanate Similar but less conspicuous alterations have also been demonstrated in the liver following administration of 3 Me DAB (17) *Herman & Fitzgerald* (10) found myelin figures and the formation of whorls and agranular endoplasmic reticulum in the pancreas following treatment with ethionine and the same authors observed similar changes in the liver as well after ethionine treatment (11) Since changes in the endoplasmic reticulum may occur in different organs following various types of noxious agents it seems reasonable to presume that these alterations are unspecific and express a general response to injury

The most striking changes in the mitochondria were ring shaped or dense intramatrical structures suggesting that an electron dense substance may have precipitated on a preformed less dense core Similar structures within cardiac mitochondria in magnesium deficient rats have been reported by *Mishra & Herman* (15) These authors presumed that the deposits consisted of calcium Intramatrical granules of this type have also been described in normal muscle mitochondria in vertebrates (13) *Peachey* (16) instilled barium or strontium ions into the toad urinary bladder and observed the development of large mitochondrial granules in the epithelial as well as the muscle cells of the bladder These granules were taken to indicate a disturbance of the water and electrolyte balance Granules of the same appearance have been produced by incubation of isolated rat liver mitochondria in media containing calcium ions and inorganic phosphate (5) On the basis of these observations it seems reasonable to consider the intra

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Figs 7–8

Fig 7 Pathologically altered smooth surfaced endoplasmic reticulum Spirals of paired membranes surround a mitochondrion At the inner and outer surfaces of the spiral the paired membranes are continuous with tubules 200–300 Å in diameter Bacitracin for 3 hr × 40 000

Fig 8 Altered mitochondria in a tubular cell from a bacitracin treated mouse (6 hr) The intramatrical granules often possess a central less dense core The edge of the granules is irregular often polycyclic or scalloped The granules measure up to 1000 Å in diameter × 55 500





Fig 9

Portion of the cytoplasm of a tubular epithelial cell treated with bacitracin for 6 hr A major part of the mitochondria contain irregular electron dense granule with a less dense core In this electron micrograph five dense bodies (Db) with heterogeneous contents are visible × 22 000

matrical granules as deposits of calcium or magnesium probably as phosphates formed as a result of altered salt and water metabolism within the tubular epithelium

In conclusion our ultrastructural observations support the concept that the primary lesion in bacitracin intoxication is restricted to the proximal tubular cells and that the proteinuria occurring in this condition is caused by decreased tubular reabsorption of protein

SUMMARY

Single injections of bacitracin 3 6 and 18 hr prior to sacrifice produced renal lesions restricted to the proximal tubules The alterations observed were a reduced thickness of the apical portion of the tubular epithelial cell increased amounts of large dense bodies accumulations of smooth surfaced endoplasmic reticulum and membraneous whorls and numerous large intramatrical dense granules within the mitochondria

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THYMIC DYSPLASIA WITH IMMUNOLOGICAL DEFICIENCY

Report of Two Unusual Cases

by

JON LAMVIK and PETER JOHAN MOE

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The characteristic clinical features of the Swiss type of hypogamma globulinaemia are reduced numbers of lymphocytes in the peripheral blood reduced amounts of serum immunoglobulins and increased susceptibility towards infections (*Glanzmann & Riniker* 1950 *Tobler & Cottier* 1958 *Hitzig et al* 1958) The patients usually develop signs of immunological deficiency in the first months of life Recurrent infections particularly in the respiratory tract dominate the clinical course and the patients usually die before 1½ years of age The characteristic pathological findings at post mortem are hypoplasia of the thymus with absence of the normal cortico medullary differentiation and a marked depletion of thymocytes In addition there is a marked cellular depletion in the white pulp of the spleen and in the lymph nodes (*Good et al* 1964)

At the Gade Institute post mortem examination has recently been performed on two unrelated infants both having dysplasia of the thymus and marked reduction of the splenic white pulp and the lymph nodes One of the patients had granulocytopenia thrombocytopenia and a haemolytic process At post mortem a hypocellular bone marrow was found The other patient had a histologically malignant lympho reticular tumour in the spleen

CASE REPORT

Case 1

A.J H a prematurely born male infant birth weight 1890 g and length 43 *cm.* The parents were unrelated the mother was 28 years old Gravida I Para I Pregnancy was uneventful until 8 weeks before term when the mother had vaginal bleedings The infant was born three weeks later and transferred to the Children s Hospital shortly after delivery because of prematurity Physical examination on admission revealed a one hour old infant having no distress and with no signs of infection Capillary haemoglobin was 18 7–18 4 *g per 100* ml leucocyte count 800 *per µl* with 8 per cent granulocytes and with 340 nucleated *red cells* per 100 leucocytes

On the fourth day of life the infant was hoarse but there was no signs of pneumonia He had, however on the following day shortly after feeding an attack of apnoea with convulsion and respiratory distress Pulmonary rales were heard

and antibiotics were given Haemoglobin was 13 4 g per 100 ml leucocytes 800 per μl and platelets 18 000 per μl Later on he had several episodes of respiratory and cardiac arrest and died 3¼ hours after the first signs of respiratory distress

Autopsy At post mortem examination performed 5 hours after death a premature infant was found without external signs of malformation

In the trachea and right bronchial tree semisolid aspirated material was present The lungs showed atelectasis with patchy haemorrhages and with a more solid consistence in the right upper lobe

The thymus was very small weight 1 5 g compared to a normal weight of 9 g The organ was pale coloured and had a myxomatous consistency

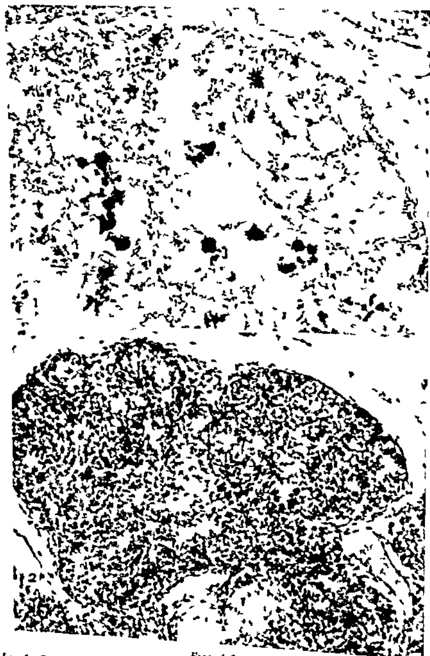
The other organs showed no macroscopic abnormality The spleen (5 g) was of normal weight No lymph nodes were found at the gross examination

The following changes were observed microscopically In the lungs acidophilic exudate was present in the terminal bronchioli and in the alveoli In addition masses of bacteria (Fig 1) which showed a positive reaction with Gram s stain were found There was practically no infiltration of inflammatory cells apart from some macrophages and nonidentified mononuclear cells in the connective tissue septa Plasma cells were not seen No inflammatory foci and no signs of bacterial invasion were found in the other organs

The thymus (Fig 2) had a lobular architecture with pale cells with an appearance like epithelial cells and reticulum cells The cells were in some areas arranged in pseudoacini No Hassal s corpuscles were found Scattered between the epithelial cells some cells with acidophilic granular cytoplasm were found The cells gave a weak PAS positive staining reaction and showed metachromasia after staining with toluidine blue Similar cells were also found in small numbers in the lymph nodes No differentiation in cortex and medulla was present and very few cells with an appearance like small lymphocytes were found between the epithelial cells

The spleen showed a normal red pulp but only a few rudimentary Malpighian corpuscles were present (Fig 3) In the mesenteric connective tissue lymph nodes with a reticular stroma were found but with a very sparse lymphocyte population The liver had a normal lobular architecture A few scattered haematopoietic foci were found in the liver sinusoids The costal bone marrow (Fig 4) was hypocellular with poorly defined leucopoiesis Megakaryocytes were fairly numerous The tunica propria and the submucosa of the small intestine contained some lymphocytic aggregates but lacking of reactive changes No abnormality was found in the other visceral organs

No abnormality was found on immuno electrophoresis of the parents sera The mother had recently born a girl infant without signs of disease



Figs 1–2

Fig 1 Case 1 Massive bacterial invasion in the lung without inflammatory response H+E, × 150

Fig 2 Case 1 Thymus with lobules of medullary tissue without Hassal's corpuscles and with very few thymocytes H+E, × 150

and antibiotics were given Haemoglobin was 13.4 g per 100 ml, leucocytes 800 per µl and platelets 18 000 per µl. Later on he had several episodes of respiratory and cardiac arrest and died 3½ hours after the first signs of respiratory distress.

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Fig 2 Case 1 Thymus with lobules of medullary tissue without Hassal s corpuscles and with very few thymocytes H+E × 150



Figs 3–4

Fig 3 Case 1 Spleen with a small and cell poor Malpighian corpuscle H+E × 3 0

Fig 4 Case 1 Costal bone marrow Hypocellular picture with poorly defined haematopoiesis Megakaryocytes fairly numerous H+E × 370

Case 2

K.M a male infant who died at the age of 7½ months His parents who were second cousins and 3 siblings were all healthy A sister died at the age of 2¼ months in the Children s Hospital from Staphylococcal septicaemia probably precipitated by urinary tract infection She had no lymphopenia and autopsy revealed no evidence of thymic dysplasia

The male infant had recurrent episodes of aphtous stomatitis from the age of 3 months and received several courses of antibiotic therapy At the age of 5 months he was admitted to the Children s Hospital. The stomatitis had cleared up but he developed signs of a gastro enteritis shortly after admission One month later while still in hospital he developed a pneumonia and the spleen became palpable The lungs had just before been normal roentgenologically He was treated with different types of antibiotics without effect Oral thrush was demonstrated and candida albicans cultured from the stools and bronchial secretions Mycostatin had only temporary effect on his oral thrush Terminally he had extensive pulmonary involvement considered to be due to moniliasis He died from respiratory failure

Leucocyte count ranged between 4 200 and 13 800 and the percentage of lymphocytes from 30 to 6 Studies performed 4 days prior to death revealed total protein of 5.5 g per 100 ml Paper electrophoresis showed no peak in the gammaglobulin region

Immuno electrophoresis examination of the serum from the parents and two brothers disclosed no deficiencies in the immunoglobulins

Autopsy At post mortem examination the lungs were found to be heavy with a solid consistence weighing three times the normal weight Grayish infiltrates were found on the cut sections The thymus (weight 4 g) was small with a lobular appearance The spleen weight was 79 g four times the normal weight The consistence was more solid than usual In the central part of the spleen a solid grayish tumour with a diameter of about 20 mm was found No lymph nodes were found on gross examination

On microscopical examination the lungs showed in all lobes a complex picture with atelectasis and emphysema The alveoli were filled with a proteinaceous material with vacuoles like those found in pneumocystis carinii (Fig 5) In addition giant cells with cytoplasmatic and some nuclear inclusions were found Granulocytic infiltrates were present but few lymphoid cells and no plasma cells The alveolar membranes and the walls of the terminal bronchioli were lined by hyaline membranes The bronchial epithelium showed extensive epidermoid metaplasia

In the thymus no cortico medullary differentiation was found Medullary lobules composed of stromal cells and epithelial cells were surrounded by fatty tissue (Fig 6) Very few thymocytes were present and no Hassal s corpuscles were seen There was some interstitial fibrosis and some acidophilic granular cells which gave a strongly positive PAS reaction were found in the interstitial tissue as well as in the medullary lobules

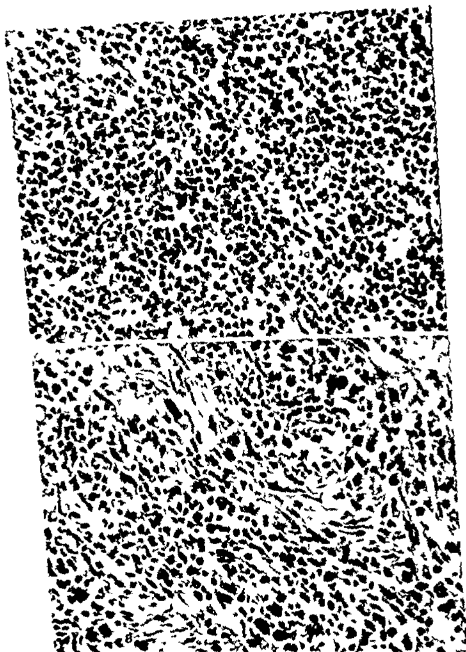
The spleen showed a prominent reticulum Lymphocytes were fairly numerous in the white pulp No germinal centres were present The centrally placed tumour was composed of lymphoid cells which in some areas had a quite uniform appearance like medium sized lymphocytes and with a general structure like a lymphosarcoma (Fig 7) In



Figs 5–6

Fig 5 Case 1 Lung with foamy material in the alveoli and moderate chronic interstitial inflammation H+E × 150

Fig 6 Case 2 Thymus with medullary lobules of stromal cells without Hassal's corpuscles The lobules are surrounded by fatty tissue without any cortical layer containing thymocytes H+E × 150



Figs 7–8

Fig 7 Case 2 Part of splenic tumour with *quite* uniform cell picture composed of cells like medium sized lymphocytes H+E × 370

Fig 8 Case 2 Part of splenic tumour with pleomorphic cell picture showing some atypical giant cells H+E × 340

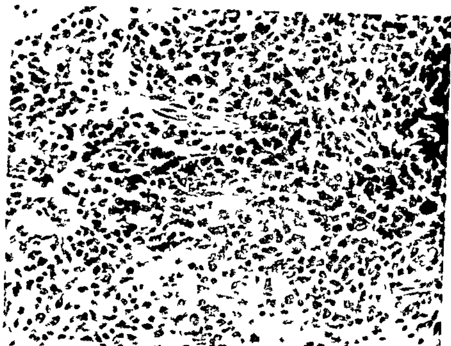


Fig 9
Case 2 Part of splenic tumour with numerous plasma cells
Methyl green – pyronin × 370

other areas the tumour showed marked variation in nuclear size and chromatin content (Fig 8) A number of giant cells were present some binucleated with prominent nucleoli In some places fibroblast proliferation with fibrosis was conspicuous In large areas of the tumour numerous cells with eccentric nuclei and strongly pyroninophilic cytoplasm were found (Fig 9) Most appeared like mature plasma cells but some were larger with immature nuclei In the central part of the tumour necrosis with calcification was present

No lymph nodes were found in the mesentery or the mediastinal connective tissue The bone marrow appeared to have a normal cellularity In the stomach duodenum and ileum very few lymphoid cells were seen and no Payer s patches noticed

No definite abnormality was found in the kidneys adrenals pancreas or thyroid Fatty degeneration was found in the liver Some small areas of liver cell necrosis were found especially *bordering* the portal tracts Some liver cells showed intranuclear inclusions appearing like bird eyes

DISCUSSION

The cases reported showed the characteristic pathological features of the Swiss type of hypogammaglobulinaemia dysplasia of the thymus and lack of proper development of the *peripheral lymphoid* organs

i e the lymph nodes and the white pulp in the spleen Both patients showed insufficient resistance against infections which caused the death in both This lack of resistance towards infections which is noticed in patients with thymic dysplasia is probably partly due to the reduced number and functional insufficiency of lymphocytes in lymphoid organs and circulating blood and partly due to deficient production of immunoglobulins Our first patient being a newborn infant was probably still supplied with immunoglobulins from the mother transferred through the placenta The second patient at 7 months of age showed a markedly reduced amount of serum gamma globulins determined by paper electrophoresis in spite of protracted infections

The first patient showed virtually no sign of cellular inflammatory response towards an overwhelming bacterial infection in the lungs This lack of cellular response is most likely explained by the leucopenia with granulocytopenia which was noticed in the blood smear on the second day of life at a time when no clinical signs of infection were present Granulocytopenia and the thrombocytopenia and haemolytic anaemia which also developed are not usually seen in the Swiss type of hypogammaglobulinaemia although several patients with severe immunological deficiency syndromes have developed bone marrow failure and at least one developed haemolytic anaemia and thrombocytopenia (*Rosen et al* 1966) In most cases the haematopoietic deficiency developed in a late stage at a time when the patients were already suffering from severe infections (*Glanzmann & Riniker* 1950 *Good et al* 1964 *Fireman et al* 1966) Therefore the infections might have been the direct cause of the bone marrow failure

In our patient an infectious cause for the granulocytopenia appears to be unlikely If an infection had been the primary cause of the marrow failure one would expect a cellular response to occur at the start of infection A cellular inflammatory picture should be present at post mortem in some areas of the lungs or in other organs However no cellular invasion was found apart from a few macrophages and other large mononuclear cells in the septa of the lungs Furthermore granulocytopenia was present soon after birth at a time when no signs of disease were noticed It appears therefore that the granulocytopenia had been the predisposing cause for the fatal pulmonary infection The terminal drop in haemoglobin and platelet count may however well be secondary to the infection

The post mortem findings in our first case were very similar to those reported by *Gitlin et al* (1964) in an infant who died at 15 days of age because of an overwhelming bacterial infection without inflammatory response A similar disorder but with complete absence of blood leucocytes in two male newborn twins was described by *de Vaal & Seynhaeve* (1959) This disorder was termed reticular dysgenesia

and the authors suggested that the thymic dysplasia may represent an incomplete form of this disorder

A graft versus host reaction induced by maternal lymphocytes passing over into the foetal circulation in the prenatal period appears to be a possibility in our first patient as well as in the case reported by *Gitlin et al* (1964) The mothers in both cases had uterine haemorrhage in the last two three weeks before the delivery which may facilitate a transfer of blood cells from the maternal to the foetal circulation A *similar* graft versus host reaction was suggested by *Hathaway et al* (1965) as the cause of a fatal pancytopenia following *transfusions* with viable leucocytes to two probably immunologically deficient infants The transfer of viable cells may only be harmful in immunologically deficient infants in parallel with the graft versus host reaction induced experimentally in immunologically deficient animals (*Porter* 1960 *Billingham et al* 1962 *Simonsen* 1962)

The second patient died following a clinical course which was quite typical of the Swiss type of hypogammaglobulinaemia The lack of cortico medullary differentiation and Hassal s corpuscles in the thymus and the lack of detectable lymph nodes and lymphoid tissue in the intestines are also characteristic of the disease No abnormality was found in the central nervous system

The most remarkable post mortem finding in this case was the presence of a grayish white tumour in the central part of the spleen surrounded by dark coloured pulp without obvious abnormality on gross examination The tumour tissue was composed of closely packed lymphoid cells The majority were larger than small lymphocytes with more irregular nuclei and more abundant cytoplasm In some areas definite variation in cell and nuclear size and chromatin content was noticed suggesting a malignant lymphoma In some parts of the tumour numerous cells with strongly pyroninophilic cytoplasm were found The majority were like mature plasma cells while some had immature nuclei and some were binucleated Lymphoid cell aggregates and plasma cells were not found in any other organs Thus the post mortem findings gave evidence of a localized malignant lymphoma with immature and mature plasma cells in the spleen of a patient suffering from thymic dysplasia with hypogammaglobulinaemia

Malignant lymphomas have been found to be associated with some immunologically deficiency states more commonly than would be expected on the basis of chance alone *Page et al* (1963) and *Reisman et al* (1963) have reported the occurrence of malignant lymphoma and leukaemia in three patients with congenital agammaglobulinaemia Lymphoreticular malignant tumours have been reported several times in patients suffering from ataxia teleangiectasia (*Boder & Sedgwich* 1958 *Peterson et al* 1964) This disorder is characterized by defects in the central nervous system a low serum content of IgA and increased susceptibility towards infections The thymus often shows

an abnormality similar to that described in the Swiss type of hypo gammaglobulinaemia

In the Swiss type of thymic dysplasia with hypogammaglobulinaemia malignant tumours have hitherto not been reported However because of the association between other forms of immunological deficiency states and malignant lymphomas it is not surprising that a lymphoma has also been found in this disorder *Schwartz et al* (1966) have recently reported a high incidence of malignant neoplasia in the lymphoid organs of hybrid mice with chronic runt disease after injection of parental lymphoid cells This observation may be an experimental equivalent to the malignant lymphomas seen in patients with immune deficiency states

The presence of numerous plasma cells in the tumour suggestive of a plasmocytoma is most remarkable Patients with the Swiss of hypo gammaglobulinaemia usually lack plasma cells However *Nezelof et al* (1964) and *Fireman et al* (1966) have reported two cases of thymic alymphoplasia with plasma cells in the lymph nodes and with the ability to produce one or several types of immunoglobulin Experimental studies in rats and rabbits have also given evidence of plasma ell differentiation in thymectomized animals *Waksman et al* (1962) howed that rats thymectomized at birth had normal numbers of lasma cells and normal concentration of gammaglobulin Yet the nimals showed depleted lymphoid tissues and failed to make anti odies after injection of bovine serum albumin (*Jankovic et al* 1962) n the rabbit absence of the thymus may lead to plasmocytosis auto immune phenomena and amyloidosis (*Sutherland et al* 1965) This finding may be relevant to the plasmocytosis observed in the splenic tumour The patient showed however no reactive plasmocytosis in the lungs which were infected with organisms which usually give a marked degree of plasmocytosis Thus the aggregation of plasma cells in the tumour appears to be an expression of neoplastic cell proliferation and not a cellular reaction induced by antigens

SUMMARY

Two infants with thymic dysplasia and poor resistance towards infections are reported One infant developed granulocytopenia thrombocytopenia and haemolytic anaemia shortly after birth No inflammatory reaction was found in the lungs where a marked degree of bacterial invasion was found The other infant suffered from protracted gastro intestinal and pulmonary fungus infections and died in respiratory failure At post mortem a malignant lymphoma with numerous plasma cells was found in the spleen No plasma cell reaction was present in the lungs which showed a chronic inflammatory reaction

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HYALINE MICROTHROMBI IN AN AUTOPSY MATERIAL

A Quantitative Study with Discussion of the Relationship to Small Vessel Thrombosis

By

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Hyaline microthrombi (HMT) (22 23 24 25) are globular eosinophilic structures which are found in the microcirculation of many organs usually unattached to the vessel wall (Fig 1) HMT have a diameter which varies between 2–3 μ and 30 40 μ They give positive staining reactions for fibrin Such structures were first reported by *Welti* (26) *Apitz* (3 4) stated that they were formed by aggregation (Koazervation) of fibrin as a result of pathological coagulation He claimed that this usually took place after death and therefore suggested that HMT were of no significance *Zink* (27) on the other hand considered them to be a special form of thrombi formed *in vivo* in streaming blood

Skjørten (25) restudied the nature of HMT Immunohistochemically they seemed to consist of fibrinogen or fibrin Ultrastructurally however they differed from ordinary fibrin thrombi by being composed of an irregular mosaic of fine filaments which only occasionally showed the periodicity of fibrin

In routine histological sections HMT are not easily seen We first observed them in fibrin stained sections from patients with disseminated intravascular coagulation (DIC) and proposed that they might be the primordial body in such conditions (23) Subsequently however we have founnd HMT in tissues from many patients without DIC Therefore it became necessary to undertake a quantitative study of the occurrence of HMT in a consecutive autopsy series The results are reported here and are compared with the results of a quantitative study of HMT in a series of cases showing various manifestations of the generalized Shwartzman reaction

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MATERIAL AND METHODS

This report is based on the study of 100 consecutive autopsies performed in the Department of Pathology Ullevaal Hospital (autopsy no 1–100/58) Case histories and autopsy reports were unknown to the author when the microscopical studies were carried out Furthermore six cases of generalized Shwartzman reaction previously reported (24) were restudied with special reference to the occurrence of HMT

Sections from all available tissue blocks were recut and subjected to microscopical examination The number of cases with sections available from the pituitary liver kidneys and lungs is indicated in Table 1 Blocks from only 12 brains were available brains were therefore excluded from the study

Autopsies had been carried out 12–58 hours post mortem Tissues were fixed in 4 per cent unbuffered formaldehyde solution and embedded in paraffin Sections were cut at 5–10 μ and stained with haematoxylin and eosin (H+E) phosphotungstic acid haematoxylin (PTAH) (16) and Martius scarlet blue (MSB) (15)

All MSB sections were systematically screened at 300 times magnification using a Reichert Zetopan Microscope with a calibrated cross table Every HMT fulfilling the diagnostic criteria stated above and having a diameter larger than an erythrocyte were counted in each section In the pituitary particular care was taken not to confuse HMT with proteinaceous material lying centrally in glandular acini because these structures frequently showed red colour in MSB stained sections similar to fibrin Only those HMT which with certainty were lying within vessels were counted

In the following conventional mural or occlusive thrombi composed of platelets fibrin or both will be called *thrombi* The occurrence of thrombi in small vessels arterioles capillaries and venules as well as the presence of precipitated fibrin in tissues was studied in PTAH sections No attempt was made to quantitate thrombi or tissue fibrin but their presence or absence was recorded

The exact outline of each section was drawn on thin cardboard cut out with a pair of fine scissors and weighed on a Mettler H 4 scale (F Mettler Zürich Switzerland) to determine the area studied in each section The number of HMT per cm^2 section area was then computed for each organ in all cases studied and will subsequently be called *density*

When the quantitative evaluation was finished all case histories and autopsy reports were studied and pertinent information regarding past and present illnesses as well as autopsy findings were recorded

The results were subjected to statistical analysis computing χ^2 with Yates modification or the Fisher exact probability test in 2×2 contingency tables (17) the Students t test (6) and the coefficient of association (24) All P values below the 5 per cent level were considered significant Two tailed tests were found suitable in all situations studied

RESULTS

1 *Consecutive Series*

Occurrence of Hyaline Microthrombi (HMT)

In the consecutive series HMT were found in 54 out of 100 cases (Table 1) More than half the cases showed HMT in the pituitary The liver kidneys and lungs showed a considerably much lower frequency of HMT (Tables 1 and 3) In other organs only occasional HMT were found

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Figs 1–2

Fig 1 Hyaline microthrombi (HMT) in pituitary sinusoid. PTAH 1160 ×

Fig 2 Conventional thrombi in alveolar capillaries (arrows) and intraalveolar fibrin formation in bronchopneumonia PTAH 240 ×


HMT

TABLE 1

Frequency and Density of HMT in Various Organs Consecutive and Shwartzman Series

Organ	Total	Frequency			Density	
		HMT cases	No HMT	P	All cases	HMT cases
Pituitary						
Consec	87	45	42	N s	30.3	58.6
Shw	5	4	1		227.0	284.0
Liver						
Consec	97	21	76	< 0.05	1.2	5.3
Shw	6	4	2		5.2	7.9
Kidneys						
Consec	97	15	82	< 0.001	0.4	2.6
Shw	6	5	1		1.6	2.0
Lungs						
Consec	95	11	84	< 0.025	0.2	1.6
Shw	5	3	2		0.4	0.8
Other						
Consec	96	3	93	< 0.0005	0.1	3.4
Shw	6	4	2		4.7	7.0
All organs						
Consec	100	54	46	< 0.10	2.3	4.3
Shw	6	6	0		8.4	8.4

Total = total number of cases with sections available P = probability of difference between occurrence of HMT in consecutive and Shwartzman series Density = number of HMT per cm² section area Consec = consecutive series Shw = Shwartzman series N s = not significant

The mean density of HMT per cm section area was 10 times higher in the pituitary than in the liver while the kidneys and lungs showed an even lower density The mean density of HMT was 2 3 for the whole series and 4 3 when cases without HMT were excluded (Table 1) 28 cases showed HMT in only one organ 13 cases in two 11 in three and two cases showed HMT in four organs

Occurrence of Small Vessel Thrombosis

Thrombi were found in small vessels—arterioles capillaries and venules— in 45 cases (Table 2) 35 of which also showed HMT Sections from the lungs were available in 95 cases Thrombi in small pulmonary vessels were found in 36 cases Among these 32 had *bronchopneumonia* and showed thrombi in the inflamed areas *frequently* at the periphery of the bronchopneumonic nodules (*Fig 2*) HMT were rare in this location Within the bronchopneumonic nodules there was usually marked intra alveolar fibrin formation In the *consecutive series* 78 per cent of the cases with bronchopneumonia showed *thrombi* in small pulmonary vessels

TABLE 2

Frequency of Small Vessel Thrombosis in Various Organs Consecutive and Shwartzman Series

Organ	Total	Frequency: Cases with thrombi	Frequency: Cases without thrombi	P
uitary				
Conse	87	9	76	N s
Shw	5	1	4	
ver				
Consec	97	2	95	N s
Shw	6	1	5	
dneys				
Consec	97	9	88	< 0 0005
Shw	6	5	1	
ings				
Consec	95	36	59	N s
Shw	5	1	4	
ther				
Consec	96	3	93	< 0 0005
Shw	6	6	0	
ll organs				
Consec	100	45	55	< 0 05
Shw	6	6	0	

Total = total number of cases with sections available P = probability of difference between occurrence of small vessel thrombosis in consecutive and Shwartzman series Consec = consecutive series Shw = Shwartzman series
N s = not significant

TABLE 3

Distribution of HMT and Small Vessel Thrombi in Various Organs Consecutive Series

	Pituitary	Liver	Kidneys	Lungs	Heart	Other	All organs
Per cent of cases showing HMT	51 7	21 6	15 5	11 6	1 1	2 8	54 0
Per cent of ases showing small vessel thrombosis	10 3	2 1	9 3	37 9	2 2	2 8	45 0

The distribution of small vessel thrombi in various organs is shown in Table 3 About 10 per cent of the pituitaries showed thrombi in sinusoids of the anterior lobe while nine per cent of the kidneys showed thrombi in arterioles glomerular capillaries and medullary veins The liver and heart showed a low frequency of small thrombi

In the consecutive series 25 cases showed small vessel thrombosis

in only one organ 14 in two organs and six cases in three organs In 27 cases small vessel thrombosis was thought to be secondary to pathological processes adjacent to the thrombosed vessels such as bronchopneumonia infarction or malignant tumours In 18 cases no such local cause of small vessel thrombosis was found These cases failed to show significantly higher frequency of bronchopneumonia than the rest of the material Furthermore the frequency of HMT was not higher in this group than in the 27 cases with small vessel thrombosis secondary to local pathological processes

Hyaline Microthrombi and other Pathological Findings

With a view to the following statistical evaluation the consecutive autopsy series was divided in cases with and without HMT The mean section area studied per case in the HMT group was 12 7 cm² and in cases without HMT 11 5 cm² The difference was not significant ($t = 1.29$ $0.20 < p < 0.30$) The entire material showed a preponderance of males and of patients above 60 years of age as was to be expected in an autopsy material The sex rate in the HMT cases corrected for the preponderance of males in the entire material was 1 25 1 In spite of difference in sex ratio between HMT cases and the entire material a comparison between the occurrence of various clinical and pathological findings in cases with and without HMT is considered permissible

Study of the case histories and autopsy records (Table 4) revealed that there was a strong association between HMT and microscopically verified bronchopneumonia significant at the 0 1 per cent level The association between HMT and thrombi in small vessels was even stronger however and significant at the 0 05 per cent level Cases with thrombi in small vessels showed a mean of 4 2 HMT per cm² section area while cases without thrombi in small vessels had a mean of 0 7 HMT per cm² section area This difference is significant at the 5 per cent level ($t = 1.96$) When cases with bronchopneumonia and malignant tumours were excluded from the material the association between HMT and small vessel thrombosis was significant at the 0 25 per cent level (Table 4) Finally when cases with small vessel thrombosis were excluded from the material bronchopneumonia showed no association with HMT (coefficient of association = 0 064)

No significant association was found between HMT and malignant tumours without bronchopneumonia When cases with bronchopneumonia were excluded there was no significant association between HMT and deep vein thrombosis arterial emboli and thromboses or atherosclerotic heart disease (Table 4) The consecutive series included only there cases of shock defined as blood pressure below 90 mm Hg systolic for more than 6 hours before death Two of these cases showed HMT and one did not

No association between the time elapsed from death to autopsy and

TABLE 4

Association between HMT and Various Autopsy Findings Consecutive Series

Finding	Per cent in cases with HMT	Per cent in cases without HMT	Coefficient of association	P
Small vessel thrombosis	64 8 (35/54)	21 7 (10/46)	0 738	< 0 0005
Bronchopneumonia	57 4 (31/54)	21 7 (10/46)	0 658	< 0 001
Malignant tumours excluded				
Small vessel thrombosis	69 0 (29/42)	0 0 (0/30)	1 000	< 0 0005
Bronchopneumonia	59 5 (25/42)	10 0 (3/30)	0 856	< 0 0005
Bronchopneumonia excluded				
Small vessel thrombosis	30 4 (7/23)	13 9 (5/36)	0 512	N s
Malignant tumours	26 1 (6/23)	25 0 (9/36)	0 003	N s
Malignant tumours and bronchopneumonia excluded				
Small vessel thrombosis	35 5 (6/17)	0 0 (0/27)	1 000	< 0 0025
Deep vein thrombosis and pulmonary embolism	47 1 (8/17)	29 6 (8/27)	0 363	N s
Arterial thrombosis and embolism	29 4 (5/17)	22 2 (6/27)	0 186	N s
Atherosclerotic heart disease	58 5 (10/17)	51 5 (14/27)	0 140	N s

Absolute numbers are given within parentheses N s = not significant

TABLE 5

Association between Small Vessel Thrombosis and Various Autopsy Findings Consecutive Series

Finding	Per cent in cases with small vessel thrombosis	Per cent in cases without small vessel thrombosis	Coefficient of association	P
Bronchopneumonia	73 3 (33/45)	14 5 (8/55)	0 884	< 0 0005
Malignant tumours excluded				
Bronchopneumonia	79 3 (23/29)	11 6 (5/43)	0 933	< 0 0005
Bronchopneumonia excluded				
Malignant tumours	58 3 (7/12)	17 0 (8/47)	0 744	< 0 025
Malignant tumours and bronchopneumonia excluded				
Deep vein thrombosis and pulmonary embolism	50 0 (3/6)	34 2 (13/38)	0 316	N s
Arterial thrombosis and embolism	33 0 (2/6)	23 7 (9/38)	0 234	N s
Atherosclerotic heart disease	50 0 (3/6)	55 2 (21/38)	—0 105	N s

Absolute numbers are given within parentheses N s = not significant

the occurrence of HMT was found The possible relationship between stasis and occurrence of HMT was studied but not subjected to quantitative analysis The finding of HMT alone or adjacent to scattered red cells was as frequent as the finding of HMT in vessels showing red cell stasis

Small Vessel Thrombosis and other Pathological Findings

Associations between thrombi in small vessels and various pathological findings are given in Table 5. *Microscopically verified bronchopneumonia* showed a strong association with *small vessel thrombi significant at the 0.05 per cent level.* When bronchopneumonia was excluded from the material, malignant tumours showed a positive association with thrombi in *small vessels significant at the 2.5 per cent level.* Many of these thrombi however were located in necrotic areas within the *tumour. Neither deep vein thrombosis* and pulmonary embolism, atherosclerotic heart disease nor arterial thrombi and emboli showed any association with thrombi in small vessels when cases with bronchopneumonia and with malignant tumours had been excluded from the material.

TABLE 6

Association between HMT and Small Vessel Thrombosis for Various Autopsy Findings. Consecutive Series

Finding	Per cent of cases with HMT showing small vessel thrombosis	Per cent of cases without HMT showing small vessel thrombosis	Coefficient of association	P
Bronchopneumonia	90.3 (28/31)	50.0 (5/10)	0.806	< 0.025
Malignant tumours excluded				
Bronchopneumonia	92.0 (23/25)	0.0 (0/3)	1.000	0.006
Bronchopneumonia excluded				
Malignant tumours	33.3 (2/6)	55.5 (5/9)	−0.429	N.s.
Malignant tumours and bronchopneumonia excluded				
Deep vein thrombosis and pulmonary embolism	37.5 (3/8)	0.0 (0/8)	1.000	N.s.
Arterial thrombosis and embolism	40.0 (2/5)	0.0 (0/6)	1.000	N.s.
Atherosclerotic heart disease	30.0 (3/10)	0.0 (0/14)	1.000	N.s.

Fisher's exact probability test is employed in all instances except for bronchopneumonia (first group). N.s. = not significant

Hyaline Microthrombi and Small Vessel Thrombosis

The association between HMT and thrombi in small vessels was studied for various clinical and pathological findings (Table 6). Cases of microscopically verified bronchopneumonia showed a positive association between HMT and small vessel thrombi *significant at the 2.5 per cent level.* When malignant tumours were excluded, the association became significant at the 0.6 per cent level. Malignant tumours without bronchopneumonia showed a negative but *not significant* association between HMT and small thrombi. When bronchopneumonia and

malignant tumours were excluded there was a positive but not significant association between small vessel thrombi and HMT in cases with deep vein thrombosis and pulmonary embolism atherosclerotic heart disease and arterial thrombosis and embolism

2 Shwartzman Series

The frequency and density of HMT in the Shwartzman series are given in Table 1 All organs showed a higher frequency of HMT than in the consecutive series The difference was significant in all instances except for the pituitaries Table 2 shows that the frequency of small vessel thrombosis was higher in the Shwartzman series than in the consecutive material There was a significant difference for kidneys other organs and all organs

The mean HMT density per cm² section area showed large variation from case to case in the Shwartzman series Cases in which the interval between the last episode of disseminated intravascular coagulation (DIC) and death covered from one to four days as judged from case histories and microscopical findings had the highest HMT density (Table 7) All cases had widespread small vessel thrombosis

TABLE 7

Density of HMT and Temporal Relationship to DIC Individual Cases Shwartzman Series

Case number	1	2	3	4	5	6
Density of HMT	1 95	0 41	31 36	6 49	7 31	2 0
Interval DIC to death days	10	½	1 2	3–4	3–4	28
Number episodes of DIC	1	1	several	several	2	1

Density of HMT = number of HMT per cm² section area
Interval DIC to death = interval from last episode of DIC to death

DISCUSSION

Occurrence of Hyaline Microthrombi

HMT were found in 54 per cent of our consecutive autopsies We are unaware of any previous study of the occurrence of HMT in a consecutive material Apitz (3) stated that HMT were a rare finding our study shows that they are not

HMT were found in more than half the pituitaries in one fourth of the livers somewhat less frequently in the lungs and kidneys but rarely in other organs Furthermore the density of HMT in the liver was one tenth of that in the pituitary the kidneys showed a still lower density of HMT and the heart showed only a few HMT Blood flow per gram tissue is high in the kidneys however (9) and lower in

the liver (13) pituitary (18) and heart (7) The different density of HMT in these organs can therefore not be ascribed to differences in blood flow

Apitz (4) found HMT most frequently in the brain We had to exclude the few brain sections present from our quantitative study It is our impression however that HMT are considerably less common in the brain than in the pituitary

In a previous study (25) we presented evidence that HMT are formed *in vivo* In the present study there was no association between occurrence of HMT and interval between death and autopsy This finding further supports our view that HMT are formed *in vivo*

Occurrence of Small Vessel Thrombosis

In our consecutive series small vessel thrombosis was found in 45 per cent of the cases Close to 40 per cent of the lungs and about 10 per cent of the pituitaries and kidneys showed small thrombi but only two per cent of the livers and hearts *Hairem* (11) found thrombi in small arteries in about three per cent of 215 hearts in a consecutive autopsy material We have been unable to find other figures on the frequency of small vessel thrombosis in a consecutive autopsy material

In our material small vessels were mainly occluded by fibrin thrombi but occasional platelet thrombi were also seen *Eeles & Sevitt* (10) made similar findings in a study of small vessel thrombosis in injuries and burns

Relationship of Hyaline Microthrombi to Small Vessel Thrombosis

In our consecutive series there was a highly significant association between HMT and small vessel thrombosis In spite of this the distribution pattern of the two types of thrombi was different with maximal frequency in different organs This could mean that conditions which lead to the formation of thrombi in small vessels promote the formation of HMT by altering the composition of the plasma so that HMT are formed in streaming blood as suggested by *Zink* (27) Subsequently HMT might tend to become caught in organs with sinusoidal blood flow or complicated vascular pattern *Zink* (27) suggested that slow blood flow favoured the formation of HMT We tried to evaluate the relationship of stasis to HMT with negative results

In our material bronchopneumonia showed a strong positive association with HMT which however rarely were found in the lungs in these cases When cases with small vessel thrombosis were excluded from the material bronchopneumonia failed to show a higher frequency of HMT than the rest of the material Furthermore cases without bronchopneumonia also showed a significant association between HMT and small vessel thrombosis These findings are a strong indication that the formation of HMT is causally related to small vessel thrombosis and not to bronchopneumonia

In a previous paper (23) we proposed that HMT might lead to progressive thrombosis after having been embolized to small vessels of various tissues. Changes which might indicate progressive thrombosis around HMT are rare, however. We have seen such changes less than ten times. *Zink* (27) discussed the possibility of progressive thrombosis around HMT, but stated that he had not observed it. *Hardaway et al.* (12) suggested that progressive thrombosis might occur around HMT, but this phenomenon is not illustration in their report.

The observation of HMT surrounded by conventional thrombi indicates that HMT are formed *in vivo*, but not necessarily that HMT have initiated progressive thrombosis. During the formation of conventional thrombi in small vessels, HMT might occasionally be formed near by and in rare instances become surrounded by conventional thrombus material.

Ashford et al. (5) found high fibrinolytic activity in veins with a diameter less than 50 µ. HMT are strongly associated with small vessel thrombosis. *Skjørten* (25) demonstrated that the ultrastructural pattern of HMT differs from that of ordinary fibrin, indicating a different mode of polymerization. *Alkjaersig et al.* (2) have shown that fibrinogen split products interfere with the normal polymerization of fibrin. Therefore, the formation of HMT might possibly be related to activation of the fibrinolytic system initiated by small vessel thrombosis.

Small Vessel Thrombosis and Bronchopneumonia

Lobar pneumonia is associated with intra-alveolar fibrin formation and extensive capillary thromboses (14, 20). We found small vessel thrombosis in 78 per cent of our cases of bronchopneumonia. Such a high frequency of capillary thrombosis in bronchopneumonia is not mentioned in most texts on the pathology of pneumonia.

In experimental DIC produced by intravenous thrombin infusion, large amounts of fibrin are found in pulmonary vessels, small and large (1). In this situation, however, there is very little or no fibrin in pulmonary alveoli. On the other hand, in bronchopneumonia intra-alveolar fibrin is formed as a part of the inflammatory exudate. Capillary thrombi are found scattered in the periphery of the bronchopneumonic nodules and not in parts of the lungs which are unaffected by the inflammatory process. This indicates that in bronchopneumonia, small vessel thrombosis in the lungs is related to the inflammation and is not a result of DIC or embolization. However, in exceptional cases (8, 24), massive pneumonia with pneumococcal septicemia may precipitate DIC.

Hyaline Microthrombi and Small Vessel Thrombosis in other Pathological Conditions

Malignant tumours showed a significant positive association with small vessel thrombosis even when cases with bronchopneumonia were

excluded but no association with HMT McKay (17) reported three cases of malignant tumours with disseminated small vessel thrombosis and proposed that thrombosis might have been precipitated by the release of thromboplastin substance from the tumour itself Our consecutive series included 28 cases of malignant tumours None of these showed disseminated small vessel thrombosis In most of our cases small vessel thrombosis was found in the tumour itself in or adjacent to necrotic areas in which there appeared to be very limited circulation This might explain the lack of association between HMT and small vessel thrombosis in our tumour cases

The consecutive series included 16 cases with deep vein thrombosis and pulmonary embolism when bronchopneumonia and malignant tumours had been excluded These cases showed neither a significant association with HMT nor with small vessel thrombosis The number of cases in each of these groups is small and does not justify any conclusion regarding the relationship of HMT and small vessel thrombosis to thrombosis in large vessels

Hardaway et al (12) found HMT in small vessels in 29 out of 3? cases of shock In our consecutive series only three cases of clinical shock were recorded two of which showed HMT One of the latter also showed small vessel thrombosis In a retrospective study like ours however many cases of shock may have escaped recognition and been included in other groups Our material gives no information on the occurrence of HMT in healthy subjects dying suddenly *Remmele & Harms* (19) found HMT in only one out of 56 cases of sudden death which indicates that HMT are rare in this condition

Remmele & Harms (19) studied 112 cases of shock and found small vessel thrombosis in 61 cases with a 50 per cent frequency in livers kidneys and lungs These figures include HMT as well as ordinary thrombi Still they show a considerably higher frequency of thrombi than that observed in our consecutive series and thus support experimental and clinical studies reviewed by *McKay* (17) which indicate that shock is frequently associated with widespread small vessel thrombosis *Eeles & Sevitt* (10) studied the occurrence of small vessel thrombosis in severe injuries and burns and found a lower thrombus frequency than the one observed in our consecutive series Technical differences may account for this apparent discrepancy

Hyaline Microthrombi and Disseminated Intravascular Coagulation

In the Shwartzman series the density of HMT varied from 0 4 to 31 1 per cm section area A high density was found in cases with evidence of two or more episodes of intravascular coagulation going on until the last days before death Two cases which showed signs of only one episode of DIC having taken place 10 and 28 days before death respectively showed few HMT This might mean that the mechanisms

which facilitate the formation of HMT were no longer active and that HMT previously formed had been eliminated from the circulation On the other hand a case of meningococcal septicemia which led to death in 12 hours had the lowest number of HMT in this series in spite of the fact that there was extensive small vessel thrombosis in glomerular capillaries adrenals and skin In this situation the mechanisms promoting the formation of HMT might still be poorly activated because of the short duration of the disease

DIC is an intermediary mechanism of disease (17) in a number of different and frequently fatal conditions in which small vessel thrombosis may vary in intensity from cases showing only a few thrombi in scattered organs to cases with a generalized Shwartzman reaction In our consecutive series 18 cases showed small vessel thrombosis which could not be ascribed to local processes adjacent to the thrombosed vessel such as inflammation and necrosis We believe that these cases may represent a low degree of DIC which therefore seems to be a fairly common event in critically ill patients The generalized Shwartzman reaction on the other hand is rare having a prevalence in our laboratory of about one per 1000 autopsies (24)

In a previous paper (23) we suggested that HMT were the first thrombi formed in DIC and that small vessel thrombosis might take place around embolized HMT The present paper has failed to support this theory We have found HMT in patients with small vessel thrombosis caused by bronchopneumonia as well as by DIC It seems that the occurrence of HMT is independent of the cause of small vessel thrombosis The presence of HMT in microscopical sections is a strong indication that small vessel thrombosis has also taken place but not necessarily in the same organ The presence of HMT in sections from several organs indicate that DIC may have taken place but is not diagnostic of this condition

SUMMARY

The occurrence of hyaline microthrombi (HMT) was studied quantitatively in 100 consecutive autopsies and in six fatal cases of generalized Shwartzman reaction HMT were found in 54 cases in the consecutive series with a mean density of 2 3 HMT per cm² section area They found in all Shwartzman cases with a mean density of 8 4 HMT per cm² section area

Small vessel thrombosis was found in 45 cases in the consecutive material 20 of these had thrombi in two or three organs Small vessel thromboses which could not be ascribed to local processes adjacent to the thrombosed vessels were found in 18 cases HMT showed a highly significant association with small vessel thrombosis and had maximal frequency in the pituitary while small vessel thrombosis was most commonly found in the lungs in relation to bronchopneumonic nodules Small vessel thrombosis was found in 78 per cent of the cases of

bronchopneumonia studied Small vessel thrombosis appears to facilitate the formation of HMT, possibly through the simultaneous activation of the fibrinolytic system

It is concluded that the demonstration of HMT is a strong indication that small vessel thrombosis also has taken place but not necessarily in the same organ HMT occur with high frequency in cases of disseminated intravascular coagulation but are not diagnostic of this condition

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QUANTITATIVE STUDIES ON CORTISOL INDUCED DECAY OF LYMPHOID CELLS IN THE THYMOLYMPHATIC SYSTEM

By

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Received 8 1 69

Several attempts to estimate the number of pyknotic cells in the thymo lymphatic system of the normal rat and mouse have been made (4 10 18 21 23) In a recent study it was shown that a high number of dead and dying lymphoid cells could be registered through the use of Dye Exclusion Test (D I T) on unfixed lymphoid organ suspensions (4) The number of dead and dying cells registered in this manner exceeded several times the number of above mentioned pyknotic cells

In the light of this observation (4) we searched for an experimental agent which would change the ratio between normal and dying lympho cytes in the thymolymphatic system It is well known that a single *injection of cortisol given to an animal or a human being* will cause a rapid fall in the number of circulating lymphocytes and a strong involution of various lymphoid organs The microscopic picture of the involution shows massive pyknosis karyorrhexis and cytoplasmatic budding of the lymphocytes These changes are followed by a rapid phagocytosis of nuclear fragments The most pronounced degree of involution takes place in the thymus (5 8 18 22 25)

The aim of the present study was by application of the D I T to investigate (1) the cortisol induced involution of the thymolymphatic system (2) the ratio of the number of pyknotic cells to the number of stained cells in the thymus during treatment with cortisol (3) the use of stained thymolymphoid cell number as a kinetic parameter in the study of cortisol induced involution and re*generation of the* thymus gland

MATERIAL AND METHOD

Male and female albino mice of the C strain were examined The animals were housed under *standard conditions* *They were separated in two groups* according to age and experimental procedure In the first group (?5 *mice* 2 months of age) each animal received 5 mg In the second group (*60 mice* 1 month of age) 1 mg of cortisol (hydrocortisoneacetate dissolved in sterile 0 9 per cent saline solution LEO) injected intraperitoneally One hour to 14 days *after* the injection 4 to 10

animals from each group were examined As control several untreated animals were examined during the experimental period

Animals were killed by cervical dislocation In the first group the thymus mesenteric and axillary lymph nodes were removed and immediately transferred to chilled Hank Solution In the second group only the thymus was removed Single cell suspensions from the various lymphoid organs were prepared and the nigrosin dye exclusion test performed as described in detail elsewhere (4) In the second group the thymus gland was weighed wet and one lobe transferred to Lillie s fixative and embedded in paraffin Six microns slices were sectioned in an ordinary microtome Slices stained in H E Feulgen and gallocyanine formed the basis for estimation of the degree of involution and the incidence of mitosis and pyknosis

Several technical factors are of importance with regard to the result of DET It is especially important that the dye concentration the staining period the density of the suspension and the temperature are kept within certain critical limits (2 4 9 20 24) Preliminarily a technique that ensures reproductibility of the DET was studied in lymphoid cell suspensions (3)

RESULTS

With present suspension techniques the ratio of free lymphoid cells to accidental fixed reticular cells is app 50 1 as decribed in detail elsewhere (4 21) In lymphoid organ suspensions from animals *treated* with cortisol the number of free lymphoid cells pr mg organ weight decreases sharply yet the ratio of free to fixed cells remains unchanged Fig 1 shows graphically the percentage of free nigrosin stained cells *in relation to the total* number of free cells in various lymphoid organ suspensions Each point represents mean percentage *of stained* cells in suspensions of thymus mesenteric or axillary *lymph nodes* from 5 to 10 animals Bars represent standard deviation *of each* sub group The abscissa indicates hours and days after injection of cortisol the ordinate indicating the percentage of stained cells

As indicated in Fig 1 a rapid increase in the *number of stained* cells from various lymphoid organs follows injection *of* 5 *mg* of cortisol reaching a maximum on the 5th day It shows furthermore the particular sensitivity of thymus *to cortisol* The number of stained thymus lymphoid cells increases 4½ times while the number of stained lymph node cells increases only 2 to 2½ times and *does not* reach control level at the end of the experimental period

Fig 2 shows variations in percentage of stained thymo lymphoid cells after injection of 1 *mg of cortisol* into mice 1 month of age The table includes relative thymus weights (*mg* thymus/g mouse) Each point represents the mean weights of all thymus glands in a subgroup In this experiment variations *during the* first 24 hours were of special interest In the first 6 hours the *number* of stained cells increases up to twice the control level At 12 hours the number reaches a minimum below the control level Then a gradual rise follows reaching a maximum on the 3rd day *On the 10th* day of examination the number of stained cells reaches control level *again* Also Fig 2 shows that relative thymus weight decreases to *about half* the weight of the control thymi during the first 6 hours Throughout the following 18 hours this weight

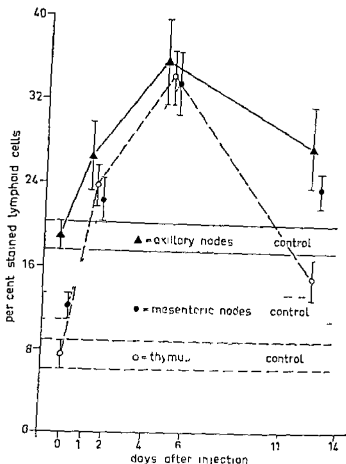


Fig 1

Cortisol induced variation in the number of nigrosin stained lymphoid cells in single cell suspensions from thymus mesenteric and axillary lymph nodes from mice injected i p with 5 mg of cortisol at time 0

remains relatively constant A decrease follows lasting from 24 hours to the 3rd day the weight reaching a minimum of app 1/4 of the weight of the control group From the 3rd day and through *thymus* weight increases but never reaches control level

Cell pyknosis and karyorrhexis occur particularly during the first day after cortisol injection appearing in thymus suspensions as well as in fixed thymus preparations Numerical estimations of cell pyknosis were based on fixed preparations where pyknoses are easily *recogniza*ble For the same reason estimation of mitotic frequency was *performed* on these fixed preparations In the 6 hour group a rise in the number of pyknoses in the thymic cortex is found The pyknoses showed diffuse distribution Also in the thymic medulla the incidence of pyknoses is slightly increased Later in the 12 hour group pyknoses are seen to gather around fixed reticular cells *filled with lymphocytic nuclear frag*

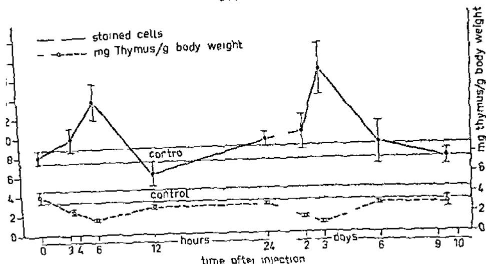


Fig 2

Change in number of nigrosin stained lymphoid cells in single cell suspensions from thymus, induced by cortisol injection Also change in relative thymus weights induced by cortisol injection

ments Still there is a tendency toward diffuse cell pyknosis in cortex and medulla In the 24 hour group pyknoses have disappeared In the cortex however a few reticular cells are visible, loaded with nuclear fragments At this time the cortex is nearly depleted of small lympho cytes

The number of mitoses during the first 12 hours does not change although many mitoses in the 6 hours group have an abnormal ap pearance At 24 hours mitotic frequency has decreased to a point where but a few immature cells are found to undergo mitosis On the 3rd day the number of mitotic cells increases strikingly From then on mitotic frequency remains at control level

DISCUSSION

According to studies by *Dougherty et al* (1964) and others (5 8 22 2a) cortisol induced injury to lymphoid cells involves both thymus and other lymphoid organs Injury is heaviest in the thymus gland how ever These studies used thymus weight cell pyknosis karryorrhexis and 17 hydroxy dehydrogenase activity in various lymphoid organs as expressions of cortisol induced involution The change in these para meters reaches a maximum shortly after injection of cortisol Accord ing to the Dye Exclusion Test however lymphoid cell decay continues and increases several days after injection of cortisol see Fig 1 Ap parently as in the above mentioned studies the thymic lymphoid cells are particularly sensitive to cortisol The effect of cortisol seems to persist since the number of stained cells continues and increases a

long time after disappearance of cortisol from the organism The half life of free cortisol in the blood of mouse is about 40 to 50 minutes thus by 100 minutes all the administered cortisol is removed (5)

The number of dead thymic lymphoid cells (Fig 2) registered by D E T does not follow the pyknotic frequency found in the fixed preparations Like others (5 8 22 25) we find a maximum degree of pyknosis within the first day after injection of cortisol

We believe that cell pyknosis and karyorrhexis both are expressions of an acute effect of cortisol on small lymphocytes while nigrosin staining mostly results from a protracted effect of cortisol on more primitive lymphocytes incl inhibition of DNA replication and inhibition of mitosis at the metaphase stage Thus we find no simple relationship between the number of cell pyknosis and the number of cortisol induced stained cells Rather the two kinds of transformation registered here appear to be independent expressions of cell decay *Meyers & Wolfe Slade* (1964) who studied the effect of X irradiation of thymic cell suspensions share this view

During the first 6 hours the decrease in relative thymus weight (from 4 mg to 2 3 mg see Fig 2) is an actual result of changes of which massive cell pyknosis and increased cell stainage are simultaneous indications Migration of small lymphoid cells into the blood stream may perhaps account for part of the thymus weight decrease (11) The following reduction in the number of dying cells shows that a major proportion of cortisol sensitive thymus cells have already disappeared This agrees with the fact that thymus weight remains nearly constant for from 6 to 12 hours From 12 to 24 hours thymus weight is kept constant in spite of a rise in the number of dying cells Considering that the number of mitotic figures is very low in the 24 hour group the increase in cell death rate must be interpreted in the light of recent studies (11 13) that show blood borne cells entering the thymus during the course of steroid or X ray induced thymus involution There is some reason to believe that these blood borne cells arise in the bone marrow since this organ remains rather insensitive to a single injection of cortisol (26) However it is possible that the observed mitotic activity in the 12 hour group may partly explain the constancy of thymus weight during the 12 to 24 hours period

The further increase in the death rate of thymus cells along with a thymus weight decrease from the 1st to the 3rd day seems to show that blood borne cells are rather short lived Cortisol induced inhibition of DNA replication and cell mitosis destroy such cells in the thymus environment Autoradiographically the short life span of the majority of the primitive thymic lymphoid cells (about 8 hours) has been shown in numerous studies (12 17 19) At the last day of the experimental period (Fig 2) the number of dying thymic lymphoid cells reaches control level At this point the thymus weight is normal and histologically the gland shows complete regeneration

SUMMARY

One and 2 months old albino mice male and female were injected intraperitoneally with cortisol The Dye Exclusion Test disclosed a striking decay of lymphoid cells in the thymolymphatic system Decay continued several days after injection The thymus was more sensitive to cortisol than various investigated lymph nodes In the thymus the percentage of stained cells increased up to 4 times the control level when the effect of cortisol was maximal the percentage of stained lymph node cells increased up to 2½ times the control level

Findings indicate that no simple relationship exists between the number of pyknotic cells and the stained cells during cortisol induced thymus involution using traditional criteria and the Dye Exclusion Test respectively Only during the first 12 hours after injection the number of pyknotic cells exceeds normal values The number of stained cells reached normal levels at the time when weight and histological appearance of the thymus was normalized

The number of dead and dying lymphoid cells measured with the Dye Exclusion Test appears to be a useful parameter in kinetic studies of lymphoid cells during cortisol induced involution and restitution of the thymus gland

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ABNORMAL BILE DUCT EPITHELIUM IN LIVER BIOPSIES WITH HISTOLOGICAL SIGNS OF VIRAL HEPATITIS

By

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Received 15 1 69

During recent years abnormal bile duct epithelium in diseases of the liver has attracted considerable attention Some workers (*Popper et al* 1962) are of the impression that it is an important readily diagnostic finding in primary biliary cirrhosis while others (*Bianchi* 1967 *Wepler & Wildhirt* 1968) though only in a smaller number of cases have described it in chronic hepatitis

The main object of the work presented here has been to obtain an impression of how frequently bile ducts with abnormal epithelium are encountered in a consecutive series of liver biopsies all showing typical changes of the parenchyma as in viral hepatitis

MATERIAL

The material consists of a total of 83 percutaneous liver biopsies all with a histological appearance as seen in viral *hepatitis* characterized by 1) focal liver cell necroses 2) condensation of reticulum fibers 3) focal Kupffer cell proliferation 4) focal infiltration with lymphocytes 5) ballooning of liver cells 6) pleomorphism of nuclei 7) mitoses and/or liver cells with two or three nuclei and 8) acidophilic bodies These changes are mainly *centrilobular* in distribution and are often associated with central vein oedema

The biopsies have been selected from a total material of 1 582 consecutive biopsies received at the pathological department Kommunehospitalet *during* the period 1 10 1963–1 10 1968

The biopsies *were obtained* by the method of *Menghini* and have been received from six medical departments (*Copenhagen Liver* Study Group) The biopsies are 1 1 4 mm thick and 1 5–4 5 cm long

The tissue has been fixed in neutral formalin and imbedded *in* paraffin. In the beginning of the period 10–1 *sections* were cut on a sledge microtome while later on 40 serial *sections* were cut on a rotary microtome The *sections* are approximately 6 μm in thickness

The assessment has been *performed* by the two authors in close cooperation without any previous knowledge of the clinical data on haematoxylin and eosin and v Gieson Hansen stained *sections* as well as on sections *stained* for reticulin

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Our thanks are due to *K Winkler* M D Head of dept for Clinical Physiology Kommunehospitalet for evaluation of clinical data

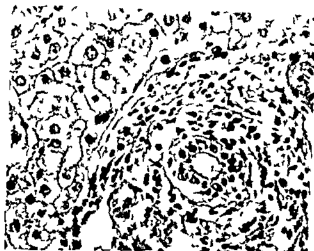


Fig 1

Portal area containing a bile duct with slightly abnormal epithelium The cells are somewhat enlarged with lightly stained partly vacuolated cytoplasm and here and there show karyopyknosis There is only a slight inflammatory infiltrate in the connective tissue consisting of lymphocytes and histiocytes × 140

In addition to bile ducts with abnormal epithelium, the degree of hepatitis (1-3 according to the number of focal liver cell necroses and the variation in size of the hepatocytes and their nuclei) and the degree of fibrosis cholestasis bile duct proliferation portal inflammation and piece meal necroses (o 3) have been recorded Cholestasis has been registered when indisputable intercellular or intracellular bile thrombi have been demonstrated

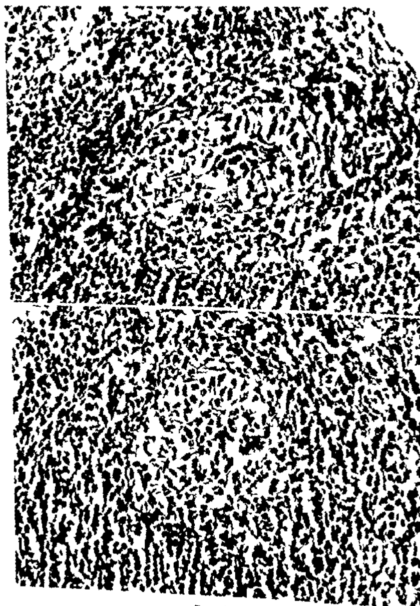
RESULTS

Abnormal bile duct epithelium has been demonstrated in 14 out of the 83 biopsies These 14 biopsies are in the following pages designated group 1 while the remaining 69 are called group 2

The epithelium of the bile ducts in the portal areas is when it is abnormal enlarged swollen with lightly coloured cytoplasm partly confluent with ill defined cellular limits and generally with areas with an indistinct basement membrane and infiltrated with lymphocytes and sometimes plasma cells and granulocytes The lumen is completely obliterated in four cases partly in three In the vicinity there is a considerable infiltration with lymphocytes and histiocytes but no necrosis or occurrence of bile pigment Only in a few cases signs of development of germinal centres are observed In eleven cases there is proliferation of bile ducts in the same portal area as in which abnormal bile ducts are seen

In serial sections it is found that the changes are segmental and often only embrace part of the circumference of the bile duct In each biopsy only one or possibly two or three abnormal bile ducts are seen

In one biopsy only quite slight changes are seen (*Fig 1*) in the others the changes have been quite pronounced (*Fig 2 and 3*)



Figs 2–3

Fig [illegible] containing a bile duct with markedly abnormal epithelium The [illegible] enlarg[illegible] and [illegible] show karyopyknosis and karyorrhexis and are in addition infiltrated by lymphocytes The lumen contains cellular [illegible] and [illegible] a pronounced infiltration with inflammatory cells [illegible] 350

Fig [illegible] with partly [illegible] at [illegible] lumen × 350

[illegible]

TABLE 1

The Table Shows the Distribution According to Sex Age and Different Histological Qualities in the 14 Biopsies with Abnormal Bile Duct Epithelium (Group 1)

Patient no	Liver biopsy no	Sex	Age	*Parenchyma*			*Portal tracts*				
				Degree of hepatitis	Degree of cholestasis	Degree of piece meal necrosis	Degree of fibrosis	Degree of inflammation	Degree of typical germinal centres	Degree of bile duct proliferation	Degree of periductal fibrosis
1	1421	♀	65	++	0	0	0	++	0	0	0
2	1430	♀	56	++	0	+	++	++	0	++	0
3	1476	♀	50	+++	0	0	+	++	0	0	+
4	1555	♀	67	+	0	+	+	++	0	+	0
5	1563	♀	48	++	0	++	+	+++	0	++	0
6	1651	♀	77	+++	0	0	+	+	0	0	0
7	1858	♀	80	++	+	+	++	++	+	+	+
8	1934	♀	46	++	0	+	++	+++	0	+	+
9	2176	♀	59	+	+	0	0	+	0	0	+
10	2304	♀	45	++	+	+	0	++	0	++	0
11	2429	♀	82	++	0	+	+	++	0	+	0
12	2717	♀	6	++	0	++	++	++	0	++	0
13	2961	♀	45	++	0	0	++	++	0	++	++
14	3101	♀	78	++	0	+	+	++	0	+	0

As far as may be judged on the basis of unaffected parts of the bile ducts the lesion has in all cases affected ducts of medium size This is further supported by the size of the concomitant branch of the portal vein and the central position of the pathological ducts in the portal areas

Table 1 shows the distribution to sex age and different histological qualities in the 14 biopsies with abnormal bile duct epithelium While all 14 biopsies in group 1 come from women the sex distribution in the remaining part of the material (69 biopsies) is 33 men and 36 women

The age distribution in group 1 is between 45 and 82 years with an average of 62 years while group 2 has an age distribution between 16 and 81 years with an average of 52 years

Only two biopsies in *group 1* show slight hepatitis while the other twelve present moderate or severe hepatitis Submassive hepatic necrosis has not been seen In three of the biopsies there is a slight cholestasis In none of these three cases is the lumen of the bile ducts with abnormal epithelium seen to be obliterated

More than half (in all nine) show piece meal necroses of which two are pronounced There is inflammation of the portal tracts in all biopsies two only in light degree and portal fibrosis in twelve In five biopsies the portal fibrosis is heavy with partial disruption of the peripheral portions of the lobules in four cases Nodular regeneration has not been demonstrated Only in one biopsy typical germinal centres are found In ten biopsies there is proliferation of the bile ducts This is pronounced in five cases Periductal fibrosis is demonstrated in six biopsies

The degree of hepatitis and the frequency and degree of piece meal necrosis portal fibrosis and inflammation as well as bile duct proliferation is greater in group 1 than in group 2 A comparison of these qualities is shown in Table 2

In *group 2* a total of 18 biopsies with cholestasis has been found Of these 16 show light and 2 moderate cholestasis None contain germinal centres and only one has periductal fibrosis Cholestasis is in all cases—both in group 1 and 2—centrilobular Bile lakes have not been demonstrated

Furthermore 64 cases with portal inflammation have been found in group 2 Five cases were without inflammatory cells in the connective tissue

In ten biopsies submassive necroses defined as larger coherent liver cell necroses are found The submassive necrosis is in all cases localized centrilobularly and there is only a slight inflammatory cell response to it In eight cases the submassive necrosis is found along with a moderate or severe hepatitis in two cases with a light hepatitis Only one case is without portal fibrosis (one of the two cases with light hepatitis)

TABLE 2

A Comparison of the Histological Qualities in Group 1 (14 Biopsies with Abnormal Bile Duct Epithelium) and Group 2 (69 Biopsies without Demonstrable Abnormal Bile Duct Epithelium)

	Degree of hepatitis			Degree of piece meal necrosis			Degree of portal fibrosis			Degree of portal inflammation				Degree of bile duct proliferation		
	+	++	+++	0	+	++	0	+	++	0	+	++	+++	0	+	++
Group 1 (14 biopsies)	2 (14%)	10 (71%)	2 (14%)	5 (36%)	7 (50%)	2 (14%)	2 (14%)	7 (50%)	5 (36%)	0 (0%)	2 (14%)	10 (71%)	2 (14%)	4 (28%)	5 (36%)	5 (36%)
Group 2 (69 biopsies)	36 (52%)	25 (36%)	8 (12%)	54 (78%)	13 (19%)	2 (3%)	25 (36%)	34 (49%)	10 (15%)	5 (7%)	48 (70%)	15 (22%)	1 (1%)	44 (64%)	19 (28%)	6 (9%)

The figures refer to the number of biopsies in each subgroup of the five qualities. In brackets is given the percentage which the number is of the total subgroup.

DISCUSSION

From our results it appears that we in 83 consecutive biopsies with a histological appearance typical for viral hepatitis have found bile ducts with abnormal epithelium in 14 cases

Five of the fourteen showed viral hepatitis without piece meal necroses Of these four had no or very slight fibrosis of the portal areas without bile duct proliferation while one had a moderate increase in the amount of connective tissue with some bile duct proliferation The remaining nine cases had a histological appearence as seen in chronic aggressive hepatitis with moderate activity and with features of acute hepatitis (*de Groote et al* 1968)

A liver biopsy is considered to be the best way to arrive at a diagnosis of viral hepatitis (*Smetana* 1963) and it must be considered overwhelmingly likely that all or nearly all the patients have viral hepatitis It is somewhat more difficult to prove that the abnormal bile duct epithelium is a part of or a sequel to hepatitis

In 20 per cent of all biopsies with hepatitis we have found abnormal bile duct epithelium and it is rather unlikely that this should be a pure coincidence

In this connection it should be mentioned that in the stated period of time it has not been the rule of the departments from which we receive liver biopsies to perform biopsies on patients with uncomplicated acute hepatitis

Furthermore 20 per cent is a minimum figure as it must be considered very likely that an additional increase in the number of serial sections from the individual biopsy supplemented by procurement of more and larger biopsies from the individual patient will result in a more frequent demonstration of the bile duct changes considered here

In none of the fourteen cases do the clinical or biochemical data suggest an affection of the larger bile ducts or a primary biliary cirrhosis The clinical condition of all the patients corresponds to subchronic or chronic hepatitis and three of the patients have later developed portal cirrhosis

Changes in the portal bile ducts of the same type as described in this article have previously been demonstrated in primary biliary cirrhosis (*Bagginstoss et al* 1964) in a few cases of chronic hepatitis (*Bianchi* 1967 *Wepler & Wildhirt* 1968) and in animal experiments following administration of α naphthyl isocyanate (*Phillips & Steiner* 1964)

Previously abnormal possibly *necrotic* bile duct epithelium in the portal areas has been considered diagnostic for primary biliary cirrhosis This seems on the basis of the above findings to be less likely and it must be assumed that some cases of hepatitis especially chronic hepatitis also involve the portal bile ducts so that some of the cases previously classified as primary biliary cirrhosis are hepatitis with abnormal bile duct epithelium

SUMMARY

83 consecutive biopsies with typical histological changes as in viral hepatitis have been examined in serial sections 14 biopsies showed abnormal bile duct epithelium with swollen and necrotic cells and with infiltration of inflammatory cells predominantly lymphocytes The changes are only seen in some portal areas They are segmental and have in 9 cases been found in biopsies with an appearence as seen *in chronic aggressive hepatitis with moderate activity*

All 14 patients are women in the age group 45 to 82 years (average 62 years)

It is rendered probable that changes of the bile ducts are part of or a sequel to hepatitis and are thus not diagnostic for primary biliary cirrhosis

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THE INVOLUTION OF THE FOETAL ADRENAL CORTEX

A Light Microscopic Study

By

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Received 29 1 69

It has been known since 1911 that the microscopic picture of the foetal adrenal cortex differs from the appearance of that of the adult organism (*Elliot & Armour* 1911)

In about six weeks old foetuses the adrenal cortex is differentiated into two clearly separated zones The foetal zone also called the x zone and the permanent zone (*Uotila* 1940) At this age the foetal zone forms about 90 per cent of the whole cortex It consists of large polyhedral cells with light acidophilic cytoplasm and large pale nuclei The cells are arranged in cords with interjacent sinusoidal capillaries (Fig 1) The permanent zone of the foetal cortex is a narrow rim of smaller cells with sparse darker acidophilic cytoplasm and small nuclei with dense cromatine arranged in formations reminding of the structure of zona glomerulosa in the adult adrenal cortex

The absolute weight of the adrenals increases throughout the foetal life In relation to the total body weight it is *greatest in the fourth* month (*Elholm & Mieminen* 1950) and in newborn infants it is 0 2 per cent of the body weight while in adult it is 0 01 per cent (*Morison* 1963)

About the time of birth the permanent zone comprises 15 to 25 per cent of the whole adrenal cortex (*Swinyard* 1943 *Fahl a* 1951) but at this time the foetal zone starts to degenerate while the permanent zone proliferates towards the centre and differentiates into the three well known zones (Fig 2)

The process of involution has been described by *Elliot & Armour* 1911 *Lewis & Pappenheimer* 1916 *Benner* 1940 *Goldzieher* 1944 *Rotter* 1949 *Lanman* 1953 *Dhom* 1965 and *Hatakeyama* 1966 but some obscurity still seems to exist about both its course in time and the nature of the process The purpose of this microscopic investigation has been to study the relation of the involution to the time of birth and to describe more exactly the morphological elements that form the involution



Fig 1

Adrenal from a foetus weighing 65 g and measuring 14 8 cm crown heel It shows the broad foetal and the small permanent zone Haematoxylin eosin staining Magnification × 140

MATERIAL AND METHODS

The present material comprises adrenals from 100 autopsies of children ranging in age from 16 weeks of foetal life up to one year after th birth The study includes adrenals from 17 stillborn and 83 live born infants 82 per cent of the infants were premature the composition of the material appears from Table 1 No attempt has been made to select cases with respect to clinical findings in mother and infant or to the diagnoses at autopsies However the adrenals from foetuses and infants of mothers with rhesus immunization and diabetes mellitus and from anencephalic foetuses have been excluded because these adrenals diverge morphologically from the group as a whole (*Miller et al* 1944 *Burne & Langley* 195f *David et al* 1963) The cause of death was hyaline membrane complex in 26 cases in 24 cases severe malformations in 5 cases trauma during delivery and in 5 cases infections More over 6 cases had kernicterus without known immunization In the remaining cases, only atelectasis mostly in connection with severe prematurit was found.

All adrenals were removed 6 to 24 hours post mortem and fixed in 10 *per cent* neutral formalin Routine haematoxylin eosin and van Gieson stained *paraffin* sections have been used in all cast Special stains have been employed in some

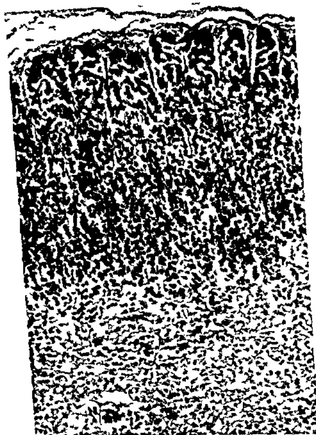


Fig 2

Adrenal from an infant who died 4½ week after the birth weighing 3100 g and measuring 52 cm crown heel The foetal zone is almost completely replaced by the permanent zone Haematoxylin eosin staining Magnification × 140

instances such as PAS fibrin staining methylviolet and Sudan Black Sudan III and oil red O in formalin fixed or unfixed freeze sections

After cutting at a right angle through the centre of the hilus sections from each specimen were estimated with respect to occurrence of inflammation haemorrhage necrosis and vacuolation in the foetal zone together with proliferation of the permanent zone

In this work haemorrhage is defined as the finding of isolated single cells com

TABLE 1

The Age Distribution of the 100 Foetuses and Infants

	Stillborn infants Foetal weeks		Live born infants Days				Months	
Age	16–25	26–40	0–1	2–4	5–8	9–14	½–3	[illegible]–12
Numbers	7	10	30	25	7	6	7	8

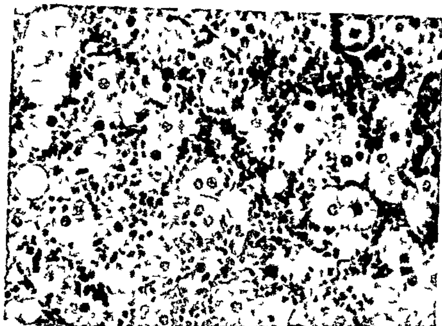


Fig 3

Haemorrhage in central part of the f etal zone Haematoxylin eosin staining
Magnification × 350



Fig 4

Cellular necrosis in the foetal zone Haematoxylin eosin staining
Ma nification × 400

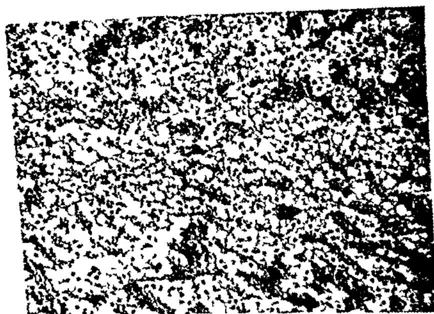


Fig 5

Vacuoles located in the central part of the foetal zone Haematoxylin eosin staining Magnification × 140

completely surrounded by erythrocytes (Fig 3) An examination of the condition of the endothelium of the capillary wall was not possible in this material The degree of haemorrhage was graded as follows (1) No haemorrhage (2) haemorrhage in the centre of the foetal cortical zone (3) haemorrhage in the whole zone, and (4) massive haemorrhage in both the foetal and the permanent zone

Necrotic cells are defined either as swollen cells with light fragmented cytoplasm and pale or entirely indistinct nuclei often combined with destruction of the cell membrane or as shrunken cells with acidophilic cytoplasm and pyknosis of the nuclei (Fig 4) Because of the finding of single necrotic cells in all cases including the smallest stillborn infants we have demanded more than [illegible] per cent of necrotic cells in every field studied with a magnification of × 400 to call a region necrotic The necrosis was graded as follows (1) No necrosis (2) necrosis in the central part (3) central necrosis combined with focal peripheral necrotic parts and (4) massive confluent necrotic parts in the whole foetal zone

The roundish clear spots which were found scattered in the foetal cortex both intra and extracellularly are described as vacuoles (Fig 5) We have chosen to study the vacuolation with a magnification of × 100 In order to quantitate this finding three degrees have been set up (1) No vacuoles (2) vacuoles located centrally and (3) vacuoles in the whole foetal zone

The growth of the permanent cortex is seen as cords of small permanent cortical cells stretching into the foetal cortex which is considered as cell proliferation of the permanent zone The width of both cortical zones was measured at a magnification of × 3[illegible] and is indicated as percentage of the total cortex. The adrenal weight is given as per cent of the total body weight.

RESULTS

The results of the examination are shown in Table 2

By the microscopic examination of the involution of the foetal cortex we never found signs of inflammatory processes in the form of infil

tration with granulocytes lymphocytes or macrophages and no deposits of fibrin

According to the above mentioned criteria of *haemorrhage this was* nearly always found in the central part of the foetal cortex Among stillborn infants in the second trimester this smallest degree of haemorrhage dominated Haemorrhage both centrally and peripherally in the foetal zone was found in nearly half of the stillborn infants in the *last trimester* in infants who died after delivery this seemed to be predominating Haemorrhage was seen during the whole process of involution and was *limited to the foetal zone* Massive haemorrhage in both the foetal and the permanent zone was only found in one case

TABLE 2

The Morphological Elements in the Involution of the Foetal Adrenal Cortex

Age	Stillborn infants		Live born infants					
	Foetal weeks		*Days*				*Months*	
	16–25	26–40	0–1	2–4	5–8	9–14	½–3	3–12
No haemorrhage (1)	1	0	3	6	2	0	0	0
Haemorrhage in the central part of the foetal zone (2)	4	4	15	11	1	1	0	0
Haemorrhage in the whole foetal zone (3)	2	6	11	8	4	5	7	8
Massive haemorrhage (4)	0	0	1	0	0	0	0	0
No necrosis (1)	5	4	14	7	1	0	0	0
Necrosis in the central part of the foetal zone (2)	1	0	5	3	1	0	0	0
Necrosis in the whole foetal zone (3)	1	5	9	11	3	3	0	0
Massive necrosis (4)	0	1	2	4	2	3	7	8
No vacuoles (1)	1	5	12	12	2	3	6	5
Centrally located vacuoles (2)	3	3	4	3	4	3	0	3
Vacuoles in the whole foetal zone (3)	3	2	14	10	1	0	1	0

Necrosis of the foetal cortex was only seen to *a slight degree* in stillborn infants In the majority of infants who died neonatally or later necrosis was *found and all infants* who lived more than one week showed necrosis This increased with time and two weeks after delivery the foetal cortex was massively necrotic and the tissue shrunken After one month the foetal cortex consisted mainly of connective tissue with single necrotic cortical cells In infants who were three months

TABLE 3

The Width of the Two Cortical Zones and the Relative Adrenal Weight in Relation to Age

	Stillborn infants				Live born infants											
	Foetal weeks				Days								Months			
	16–25		26–40		0–1		2–4		5–8		9–14		½–3		3–12	
	Foetal zone	Permanent zone	Foetal zone	Permanent zone	Foetal zone	Permanent zone	Foetal zone	Permanent zone	Foetal zone	Permanent zone	Foetal zone	Permanent zone	Foetal zone	Permanent zone	Foetal zone	Permanent zone
idth in per ut of the tal cortex	85	15	84	16	82	18	79	21	75	25	58	42	37	63	13	87
Weight in per cent of body weight	0.36		0.25		0.29		0.29		0.21		0.21		0.16		0.09	

old the foetal cortex was only a narrow rim between the permanent cortex and the medulla and had not totally disappeared even at the age of one year

In the foetal zone in some of the stillborn infants we found both central and peripheral vacuoles From delivery up to five days of age the vacuoles were mainly located peripherally and later on the few to be found were located centrally exclusively Lipid staining of adrenals from the neonatal period showed a distinct lipid accumulation corresponding to the vacuoles The staining intensity did not seem to vary with the different methods employed

Cell proliferation of the permanent cortex was not found in stillborn infants at a gestation age less than 25 weeks After birth growth was seen to increase concurrently with the involution of the foetal cortex At mid term the foetal zone constituted 85 per cent of the total cortex At birth it was 84 and 82 per cent for the stillborn and live born infants respectively Two weeks later the foetal zone was 58 per cent 3 to 12 months later it was only 13 per cent and was seen as a narrow rim

The relative adrenal weight was slowly decreasing before delivery but very rapidly after In the period from the 16th to 25th week of foetal life the adrenals constituted 0 36 per cent of the total body weight at birth approximately 0 3 per cent and one year after only 0 09 per cent (Table 3)

DISCUSSION

The results of the present study are in accordance with the findings by *Uotila* (1940) and *Ekholm & Nieminen* (1950) showing that the rela

tive adrenal weight is greatest in stillborn infants at a gestational age of less than 25 weeks. At this time the adrenals in our material constituted about 0.4 per cent of the total body weight while at birth they constituted about 0.3 per cent. *Tahka* (1951) showed that there was no difference between the relative adrenal weight of premature and mature infants. This point could not be examined here as the premature infants dominated in our material.

In the litterature there is some disagreement as regards the question whether haemorrhage or only hyperaemia is occurring during the involution of the foetal zone which is so richly supplied with sinusoidal capillaries. In light microscopic studies of an autopsy material the capillary endothelium can not be examined. Electron microscopic studies of rat and human adrenals have shown however that the endothelium of the sinusoidal capillaries is apparently incomplete (*Ross* 1962, *Luse* 1967) and that cytoplasmatic fragments can be seen in the sinusoidal capillaries during the involution (*Hatakeyama* 1966). Based on the criteria used in this work, the haemorrhage in the foetal zone is an obligatory and characteristic element in the involution. This haemorrhage starts centrally in the foetal zone already *in utero*, while at birth it includes the whole zone. We saw such haemorrhage in all the adrenals studied. A massive haemorrhage both in the foetal and the permanent zone was only seen in one case. In contrast to the supposed physiological haemorrhage in the foetal zone, haemorrhage in the permanent zone should be considered a pathological finding, possibly corresponding to findings in the clinical syndrome of adrenal haemorrhage in the newborn described by *Coldzieher & Gordon* (1932). Many authors (*Elliot & Armour* 1911, *Goldzieher* 1944, *Morison* 1963, *Dhom* 1965) doubt that the hyperaemia or haemorrhage limited to the foetal zone is a pathological phenomenon but do not histologically distinguish sharply between this constantly appearing physiological haemorrhage and the pathological one.

The occurrence of necrosis in the foetal zone is shown to be an equally characteristic and obligatory element in the involution. The necrosis begins at the same time as the haemorrhage. It may start prenatally and is pronounced shortly after birth. This sequence of events is in accordance with the observations by *Benner* (1940) and *Dhom* (1965) and should be regarded as the normal course of the necrosis. In infants aged one year we found the foetal zone shrunken and almost completely replaced by connective tissue. *Lewis & Pappenheimer* (1916) have shown that the necrotic remnants have not disappeared entirely until after the third year.

In this material vacuoles located centrally and peripherally in the foetal cortex were found in some of the stillborn infants. Just after birth vacuoles were found widespread in the whole foetal cortex in a large part of the infants who died within five days after birth. Later fewer or no vacuoles were found at the same time as necrosis and

haemorrhage became more marked. This lipid accumulation in the foetal cortex is perhaps the first sign of involution and may be considered as an expression of the interrupted hormonal connection with the placenta. In contrast to the present findings *Dhom* (1965) has found numerous—mainly centrally located—lipid vacuoles throughout the foetal life and he calls this central region a zone of lipid degeneration.

We found no cases of inflammatory changes in connection with the physiological process of involution.

On the basis of the literature and our own examination it seems justifiable to characterize the involution as a haemorrhagic necrosis without inflammatory reaction starting before birth but chiefly setting in within the first two weeks of extra uterine life.

It was shown by *Nerup et al.* (1969) that both the foetal and the permanent zones of the adrenal cortex contain antigens against which anti adrenal humoral and cellular hypersensitivity can be demonstrated in the idiopathic type of Addison's disease. This kind of anti adrenal immunity is seen only in this condition. The demonstrated haemorrhagic non inflammatory necrosis of the foetal zone with following resorption might expose the immunological apparatus of the individual to a sudden massive antigen stimulation at a time when immunological tolerance can be induced in man (*Cohen et al.* 1963, *Hutchinson et al.* 1967, *Jones* 1968). This hypothetical enhanced tolerance may explain why anti adrenal auto immunity is a very rare phenomenon.

SUMMARY

A histological study of 100 adrenal glands was carried out in order to elucidate the involution of the foetal adrenal cortex, its relation to the time of birth and the components of the process.

Necrosis and haemorrhage are considered as obligatory elements in the involution of the foetal zone. Necrosis seems to begin centrally around the time of birth. After a few days the whole zone is included. In the same period the local circulation increases and haemorrhages appear. Haemorrhage of the permanent cortical zone was seen in only one case in our series and must be considered as a pathological finding. The vacuolation of the foetal cortical cells which was shown to exist already *in utero* increased in intensity around the time of birth but disappeared completely in the course of five days. This probably represents the first sign of involution. The foetal zone rapidly shrinks after birth and concurrently the relative weight of the adrenal decreases showing that the necrotic products are resorbed. At the same time the permanent cortex proliferates and at 3 to 12 months after birth it comprises nearly the whole adrenal cortex.

No sign of inflammation in connection with the involution has been found.

Finally it is discussed if the involution of the foetal cortex which

can be regarded as a haemorrhagic non inflammatory necrosis might induce a state of enhanced immunological tolerance to adrenal tissue antigens explaining why an auto immune reaction against adrenal tissue is such a rare phenomenon

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THE CYTOTROPHOBLASTIC CELL COLUMNS AND THE CELL ISLANDS OF THE NORMAL HUMAN PLACENTA

A Light and Electron Microscopic Study with Particular Reference to the Nature of the Intercellular Material

By

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Received 4 ii 69

In the normal human placenta columns of cytotrophoblastic cells mostly covered by syncytium extend from the terminal portion of some of the villi They either end free or are attached to the basal plate forming the so called anchoring villi The columns are said to be present mainly in early months of pregnancy but remnants of cytotrophoblastic cells of anchoring villi persist until term (3)

The cell islands are rounded isolated collections of cells partly covered by the syncytium Occasionally fibrous villi are seen within the islands The cell islands are found throughout the placenta from early stages of pregnancy until term (3) Their origin has been much discussed

The cells of the columns and particularly those of the cell islands are separated by an intercellular material which has been designated fibrinoid (4 11 12 13 44) but its nature has not been clearly established *Grosser* (13) and *Wislocki & Bennett* (44) regarded it as a secretion product of the trophoblastic cells admixed with fibrin of maternal origin whereas *Boe* (2) maintained that it was transformed foetal tissue

Deposits which by light microscopy have a similar appearance are seen on the syncytium of villi and chorionic plate and on the surface of the basal plate towards the intervillous space (Rohr s stria) as well In previous papers this material was shown to be of thrombogenic origin (21 22 27) Furthermore deposits are found between the individual cells of the cytotrophoblastic shell and in the junctional zone between the trophoblast and decidua (Nitabuch s layer) The inter

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cellular material of the cytotrophoblastic shell was found to be composed of cellular debris fibrin and granular masses assumed to represent a mixture of plasma proteins aged fibrin and a secretory product of the cytotrophoblastic cells (21 22) Nitabuch s layer consisted of cell debris fibrin and granular masses probably representing precipitated fibrinogen or aged fibrin (21 22) Thus the deposits in the placenta studied so far seem to be of different origin dependent on localization and cellular relationship

The present study concerns the nature of the intercellular deposits of the cytotrophoblastic cell columns and that of the cell islands which have been investigated by light and electron microscopy The deposits may be derived from the maternal blood or alternatively they represent secretory products of the placental cells Therefore morphological signs of secretory activity in the cells of the columns and islands will be of particular interest Further evidence will be presented which may throw light on the origin of the cell islands It will be shown that the cell islands in the immature stages are developed from the cytotrophoblastic cell columns and remain attached to those The described cell islands in the mature placenta may possibly be cross sections of the placental septa

MATERIAL AND METHODS

Thirty three mature and 26 immature placentas were examined by light microscopy only The immature placentas were obtained from physically healthy women 8 to 24 weeks pregnant (menstrual age) and removed by curettage or minor caesarean section The pregnancies were interrupted on psychiatric indications

Three mature and 3 immature placentas of 10 13 and 24 weeks gestation were examined by both light and electron microscopy

Light microscopy Immediately after delivery or operation the placentas were gently rinsed in 0 9 per cent NaCl solution and cut in thin slices Specimens were taken from the central and the peripheral parts of the placentas and fixed in buffered formaline Zenker s or Helly s solution dehydrated and embedded in paraffin The following stains were used

(1) Haematoxylin azo phloxine (HP)
(2) Lendrum s Martius scarlet blue method (MSB) (20)
(3) Mallory s phosphotungstic acid haematoxylin method (PTAH)

Serial sectioning was performed of tissue blocks from 5 mature and 5 immature placentas

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Figs 1–3

Fig 1 Placenta at 17 weeks Cytotrophoblastic cell column continuous with villus The cell column is partly covered by the syncytium (arrow) The cells most distant from the villus appear vacuolated V villus MSB × 200

Fig 2 Placenta at 17 weeks Higher magnification of Fig 1 Many of the cells (8 12 microns) are vacuolated The nuclei are large and have a distinct nucleolus Pyknotic nuclei are seen MSB × 1 200

Fig 3 Placenta at 13 weeks Cell column Cells with abundant glycogen like granules (GL) Note vacuoles (VA) in glycogen areas Intercellular space (I) with granular masses and glycogen Large arrows indicate apical parts of cells Small arrows indicate lateral part of cells × 5 400


1
2
3
V
GL
VA
GL
VA
GL

Electron microscopy Tissue blocks from the basal middle and upper parts of the placenta were removed immediately after delivery or operation and fixed for 1½–2 hours in chilled isotonic 1½ per cent glutaraldehyde in M/20 phosphate buffer (pH 7 4) postfixed for 1½ hour in 1 per cent isotonic osmic tetroxide (5) and embedded in Epon 812 One micron thick sections were cut on a Huxley ultramicrotome (Cambridge Inst Co) and stained with 0 2 per cent toluidine blue for orientation by light microscope Suitable areas were selected and ultrathin sections stained with uranyl acetate and lead citrate (32) The preparations were examined in a Zeiss EM-9 electron microscope

RESULTS

Cell Columns

In the immature placenta the cytotrophoblastic cell columns (Fig 1) were composed of oval to polygonal cells of moderate size (8–12 microns) often with a large nucleus and a distinct nucleolus (Fig 2) Near the stroma of the villus the cells were closely packed with few vacuoles in the cytoplasm With increasing distance from the stroma of the villus increasing number of cytoplasmic vacuoles were seen (Fig 1) Many of these cells appeared empty but shrunken cells with pyknotic nuclei were also encountered In this location the intercellular space was larger and often filled with an eosinophilic granular to hyaline material With MSB it stained blue and with the PTAH method brownish red

In general the cell columns were covered by a layer of syncytium In some areas the syncytium was interrupted leaving the intercellular space apparently in direct communication with the intervillous space

Ultrastructurally (Figs 3 and 4) the cytoplasm of the cytotrophoblastic cells contained moderate amounts of granular endoplasmic reticulum free ribosomes and mitochondria of intermediate size

A well developed Golgi complex (Figs 4 and 4 insert) mainly located in the juxtanuclear zone was found in many of the cells The

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Figs 4–6

Fig 4 Placenta at 13 weeks Cell column Lateral aspect of the cell The cell exhibits Golgi complex (C) mitochondria (M) some glycogen like granules (arrows) and large vacuole (VA) Nucleus (N) at upper right Desmosomes (D) connecting neighbouring cells at left Tight junctions are not seen × 17 500

Fig 4 *(Insert)* Placenta at 13 weeks Cell column Golgi complex with saccules (arrows) containing material of moderate electron density Numerous vesicles containing same materials Smooth surfaced sacs (S) containing dense material are seen adjacent to Golgi complex Free ribosomes are seen × 31 000

Fig 5 Placenta at 13 weeks Cell column Peripheral region of cell showing irregularly folded apical cell membrane resembling microvilli Free ribosomes and filaments are seen in the cytoplasm Arrows probably secretion vacuoles E granular endoplasmic reticulum I intercellular space × 36 000

Fig 6 Placenta at 13 weeks Cell column Intercellular space with granules probably representing cell debris Glycogen like granules (CL) and unspecific granular masses (GR) are also seen C cytotrophoblastic cell × 14 000


VA
M
M
S
E
E
E
C
C
C

Golgi complex consisted of the usual arrays of closely packed smooth membranes forming flattened cisternae with saccular dilatations (Fig 4 insert) Usually the Golgi complex contained a homogeneous granular material of low or moderate electron density Vacuoles containing a similar material were found in the neighbourhood of the Golgi complex (Fig 4 insert) Further in the area of the Golgi complex there were smooth surfaced sacs which had an irregular shape and contained material of lower density than that in the vacuoles described above (Figs 4 and 4 insert)

Near the cell border (Fig 5) many vacuoles contained a granular material with the same electron density as that of the intercellular space

In many cytotrophoblastic cells there was a great abundance of granules (150–400 Å) single or arranged in rosettes (Figs 3 and 4) probably representing glycogen (31) Occasionally the granules occupied the entire cytoplasm except for the peripheral parts In the regions rich in granules empty vacuoles were a frequent finding (Figs 3 and 4) some were limited by a thin single unit membrane others were not

The apical cell membrane was irregularly folded and formed microvillus like projections into intercellular lakes these lakes were limited by neighbouring cytotrophoblastic cells (Fig 3) The lateral cell membrane formed mainly an even smooth outline of the cell and showed scattered desmosomes (Figs 3 and 4) but complete junctional complexes (10) were not seen The lateral intercellular spaces had a width of approximately 200 Å and appeared to communicate with the intercellular lakes

The intercellular lakes contained a finely granular material of medium electron density patchily distributed usually close to the cell membranes (Fig 3) Frequently the material was seen in the lateral intercellular space as well (Fig 4) The amount of this material increased with advancing distance from the stroma of the villus The

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Figs 7–11

Fig 7 Placenta at 13 weeks Isolated cell island Serial sections showed continuity with the central cell masses shown in Fig 8 Arrows fibrous villi HP × 75

Fig 8 Placenta at 13 weeks Cell columns originating from villi (V) Considerable amounts of intercellular material most distant from villi HP × 75

Fig 9 Placenta at 17 weeks Cell island The cells (15–30 microns) possess vacuoles mainly located peripherally Intercellular masses well developed (arrows) MSB × 1 200

Fig 10 Placenta at 14 weeks Cell island with well developed intercellular material At periphery fibrillary material stained bluish black (appears black) Fibrous villi (V) at upper left PTAH × 200

Fig 11 Placenta at 14 weeks Proliferating cytotrophoblastic cells from a terminal villus (V) Considerable intercellular material most distant from villus HP × 200


V
V
8
7
10
V

ultrastructural appearance of the granular material was similar to that within the cytoplasmic vacuoles located near the cell border (Fig 3) to that within the vacuoles found adjacent to the Golgi complex and to the content of the Golgi complex itself (Fig 4 insert) The intercellular granular material was often mixed with clusters of glycogen like granules and cellular debris (Fig 6) Fibrin was not observed

In the mature placenta the cell columns were not present remnants of cytotrophoblastic cell collections were seen only near the basal plate none of these cells were available for electron microscopy

Cell Islands

Immature placentas During the study of single sections by light microscopy typical cell islands were seen as more or less rounded isolated collections of cells (Fig 7) Sometimes villi with a fibrous appearance were found within the islands (Figs 7 and 10) The cells showed histological similarities to cells of those parts of the cell columns which were away from the stroma of the villus as in the latter cells the cytoplasm of the island cells often appeared vacuolated (Fig 9) However they were mostly larger (15–30 microns) than the column cells and the shape showed greater variation Shrunken cells with fragmented cell membranes and pyknotic nuclei were also seen

The cells were separated by an intercellular material which increased in amount with advancing gestational age In the light microscope this material appeared as finely granular or nearly hyaline masses containing small vacuoles (Fig 9) In the peripheral areas of the islands near the intervillous space the intercellular material had a more fibrillar appearance

In the first trimester the intercellular material usually stained blue with the MSB method Later in pregnancy the material particularly in the peripheral regions stained red as fibrin Occasionally a red stain

Figs 12–16

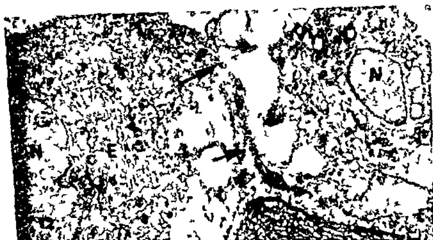
Fig 12 Placenta at 12 weeks Cell island Well developed granular endoplasmic reticulum (F) and empty area in the cytoplasm N nucleus Arrows intercellular space At lower part syncytial cell at the surface × 6 400

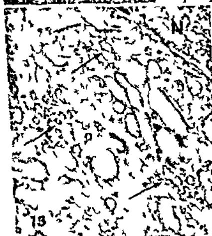
Fig 13 Placenta at 12 weeks Cell island Clusters of small vacuoles containing a material of moderate density (arrows) Granular endoplasmic reticulum are seen Note fine filaments Nucleus (N) at upper right × 36 000

Fig 14 Placenta at 12 weeks Cell island Large vacuole with clusters of fine granular masses (CR) At lower left granular endoplasmic reticulum and free ribosomes N nucleus × 18 000

Fig 15 Placenta at 12 weeks Cell island S syncytium C cytotrophoblast Granular deposit mostly located near cytotrophoblastic cell membrane × 36 000

Fig 16 Placenta at 12 weeks Cell island Intercellular material comprising cell debris granular masses and fibrils suggestive of fibrin Altered cytotrophoblastic cell (C) to the right × 18 000


N
E
N


N
13


N
14


S
15
C


C

was apparent throughout the masses With the PTAH method most of the intercellular material stained brownish red in the periphery the fibrillary component took the bluish black colour characteristic of fibrin (Fig 10) Occasionally traces of bluish black material were seen in the interior of the islands (Fig 10)

In longitudinal or slightly oblique sections of cell columns the cells most distant from the villus were histologically similar to cells of isolated islands (Fig 11) In an intermediary zone between the typical column cells as described above and the typical island cells the cells formed tightly packed clusters In areas most distant from the stroma of the villus the cells were separated by intercellular material the amount of which seemed to increase with advancing distance from the stroma of the villus

In serial sections the apparently isolated cell islands (Fig 7) were seen to originate from the distal end of cell columns as *protruding cell collections* (Fig 8) which however had a structure that was distinctly different from that of the cell columns The designation cell island may still be used as a descriptive term

Electron micrographs of the cells of the islands revealed that their structure differed from that of the cells of the columns especially from those near the stroma of the villus The endoplasmic reticulum of the island cells was more prominent (Fig 12) and often formed dilated cisternae Free ribosomes were more numerous The Golgi complex was more extensively developed and more numerous in each cell Vesicles (Fig 13) similar to those found in the cells of the columns were seen in the neighbourhood of the Golgi complex Occasionally large lipid granules were encountered The island cells contained large empty vacuoles (Fig 12) like those of the column cells Towards the periphery of the vacuoles small clusters of fine granules were frequently seen (Fig 14)

Figs 17–21

Fig 17 Placenta at term Cell island with considerable amounts of intercellular material Fibrous villus inclosed into the masses (arrow) HP × 75

Fig 18 Placenta at term Same cell collection as seen in Fig 17 as it appears deeper in the tissue block Apparently typical placental septum in connection with the basal plate (B) HP × 75

Fig 19 Placenta at term Cell island Intercellular material mostly located at superficial parts stained red (appears dark grey) Fibrous villi (arrows) at periphery and at inside of island MSB × 75

Fig 20 Placenta at term Cell island Well developed Golgi complex (arrows) and abundant vacuoles in relation to the Golgi complex F granular endoplasmic reticulum N nucleus M mitochondria At lower part intercellular space with dense granular masses × 16 000

Fig 21 Placenta at term From superficial part of cell island Intercellular material containing fibrin (arrows) and uncharacteristic granular masses × 18 000


17
18
B
N
19
21

In the cells of the islands differentiation between lateral and apical cell membranes was more *difficult* as the intercellular spaces were wider Occasionally a narrow intercellular space and desmosomes were seen similar to what was observed in the lateral aspects of the columnar cells

Many of the cells particularly in the central part of the islands showed signs of damage their cytoplasm was either darker or lighter than usual their organelles were partly lost and the peripheral cell membrane was fragmented

The intercellular space was partly filled with cellular debris and a finely granular material The latter was mainly seen close to the peripheral cell membrane and had an appearance similar to the content of the vesicles of the Golgi complex In the space between the peripheral syncytium and the underlying island cells the granular masses were mainly located close to the cell membrane of the cytotrophoblastic cells and only traces were seen adjacent to the syncytium (Fig 15)

In the peripheral part of the islands fibrils arranged in thick bundles with a higher electron density than the granular material were frequently found (Fig 16) The fibrils were similar to fibrin although characteristic cross striation was not with certainty encountered in the sections available Collagen or amyloid fibrils were not found

Mature placentas By light microscopy the cells of the islands (Fig 17) in the mature placentas were similar in shape and size to those of the immature ones but vacuolization was less developed The nuclei were oval shaped relatively large moderately dense and had a distinct nucleolus In the central parts of the islands smaller cells with small rounded dark nuclei were often encountered Cells showing definite signs of damage were more rarely seen in the mature than in the immature placentas the ones which did occur were most frequently located in the central parts of the islands

The cells were mostly separated by an intercellular material with a structural appearance and staining reactions similar to those of the cell islands of the immature placentas (Fig 19) Large apparently empty intercellular spaces (pseudocysts (15)) were often seen within the islands

In serial sections the seemingly isolated cell islands appeared to be cross sections of placental septa (Figs 17 and 18)

Electron microscopy of the cells (Fig 20) revealed a well developed granular endoplasmic reticulum and free ribosomes The mitochondria were of intermediate size Glycogen like granules were *infrequent* The Golgi complex was prominent and vesicles similar to those in the island cells of the immature placentas were seen in the neighbourhood of the Golgi complex The peripheral cell membrane was folded

The cells were most frequently separated by varying amounts of intercellular material mostly composed of cellular debris and granular masses In the periphery of the island there were in addition occasional

undles of electron dense fibrils with cross striation showing a periodi ity of approximately 230 Å indicative of fibrin (Fig 21) Uncharacter stic filamentous structures were also seen whereas collagen fibres or amyloid filaments were not observed

DISCUSSION

The origin of the cell islands of the normal human placenta has been much disputed a foetal (2 3 28 39 43 44) and a maternal (11 15 17 19 42) origin of these structures have been suggested

This study indicates that cell islands in the *immature* placenta may be developed from the cytotrophoblastic cell columns This is in accordance with *Boe* (2) who believed that the islands in the immature placenta were formed by localized proliferations of the cytotrophoblastic cell columns The strongest argument for a maternal origin is the demonstration by *Klinger & Ludwig* (19) that the *island cells* in the placentas from two immature male embryos showed female sex chromatin pattern However I have been unable to confirm these results (25) Both by light and electron microscopy the cells of the islands showed a great resemblance to the cytotrophoblastic cell columns Further they appear similar to cells of the cytotrophoblastic shell described by others (3 7 21 28 44) The decidual cells (6 7 21 45) have a different appearance

Some differences however do exist between the cells of the islands and those of the cell columns notably the more abundant Golgi complex and endoplasmic reticulum of the former This may indicate a differentiation of the island cells toward a more highly developed secretory activity On the other hand the cells of the columns are actively proliferating cells (7 24 28 40 46) whereas no signs of mitotic activity are found in the cells of the islands (24) With advancing gestational age the proliferating activity of the cytotrophoblastic cells decreases

In the *mature* placenta the cells of the islands had a cytoplasmic structure similar to that of the cells in the cytotrophoblastic cell columns and cell islands in the immature placenta Serial sections showed that the islands seemed to be cross sections of placental septa The opinions concerning the origin of the cells comprising the septa are diverging *Stieve* (38) and *Wilkin* (43) suggested that islands composed of cytotrophoblastic cells might fuse to form the septa Others have described septa as entirely composed of decidual cells (15 19 34 42) This conclusion has partly been based on sex chromatin studies (19) However similar studies (25 35) have given results indicating a dual composition of the cells This view is also maintained by *Boyd & Hamilton* (3) who found many of the cells with cytological features of the cytotrophoblastic cells The present study cannot decide whether

the island cells in the mature placenta are of cytotrophoblastic origin or represent a special development of the decidual cells

Ultrastructurally unspecific granular masses are found intercellularly in all locations investigated Two possible sources from which the intercellular masses may be derived are obvious the constituent cells of the columns and islands and the maternal blood in the intervillous space

All the cells showed numerous vesicles in relation to a well developed Golgi complex and to other parts of the cytoplasm This strongly suggests a secretory activity because a prominent Golgi complex is especially observed in secretory cells (30) and the secretory vesicles arise by separation of saccular dilatations from the Golgi complex (8) A further evidence of the secretory nature of the vesicles is the structural similarity of the content of the vesicles and that of the saccular dilatations of the Golgi complex In the pancreas (29 37) and in the pituitary (9) similar secretory vesicles condensate into larger zymogen granules A transformation of this kind was not seen Neither was zymogen granula production observed in previous studies of placental cells in human subjects (1 14 21 33 36 41) or rat (18)

The granular masses in the intercellular space showed the same appearance and density as the material in the dilated saccules of the Golgi complex and in the vesicles related to the Golgi complex and beneath the cell membrane It is therefore possible that secretory vesicles are released from the Golgi complex move towards the surface of the cell and drain their contents into the intercellular space This is in agreement with *Boyd & Hamilton* (3) who found secretory activity of the cells of the columns in their electron micrographs and interpreted the intercellular fibrinoid as a possible secretory product of the cells

The granular masses may also partly derive from maternal plasma because immunofluorescence studies have shown the presence of fibrin/fibrinogen and albumin in the intercellular deposits (22) Fibrin and fibrinogen which are immunologically indistinguishable were more extensively deposited than albumin although more fibrin/fibrinogen generally was found near the surface of the deposits towards the intervillous space than in the inner parts (22) In the present study fibrils with the ultrastructural characters of fibrin were found peripherally in the intercellular space of the islands The obvious source of this fibrin would be circulating maternal plasma fibrinogen which is easily precipitated The preferential localization of fibrin and fibrinogen at the periphery of the islands was also confirmed by the MSB and the PTAH staining methods However these methods do not differentiate between fibrin and fibrinogen (26) Further aged fibrin may take on the appearance of granular masses (27)

The intercellular space also contained glycogen like granules abundantly present in the cytotrophoblastic cell columns near the villus base Similar granules occurred also within the cytoplasm especially in

the cells of the columns It is likely that the intercellular granules are derived from the intracellular ones The granules are probably true glycogen since histochemical studies have shown small clumps of PAS positive material that could be digested by diastase both intra and extracellulary (23) The large vacuoles most frequently encountered in the cells of the islands in the immature placenta contained small amounts of granular masses or a few glycogen like granules but their significance is unknown

With increasing distance from the stroma of the villus cellular debris was seen in the intercellular space of the cell columns and islands of the immature placenta This is in agreement with the theory of *Boe* (2) that the cells may transform into the masses

Although desmosomes were often encountered especially in the columnar cells tight junctions (10) were never observed Thus it seems to be no obstacle to the transport of fluid through the intercellular spaces of the cells studied On the other hand tight junctions are variably present in the amniotic epithelium (16) in the X cells in septal cysts they are uncommon (34)

On the basis of the available information one may try to reconstruct the following life history of the cell columns and cell islands The cell columns develop from localized proliferations of cytotrophoblastic cells From these columns the cell islands develop as masses of non proliferating cells increasing in size by apposition of new cells from the proliferating cells of the columns and junctional zone As the cells move away from the zone of proliferation increasing amounts of intercellular material appear between the cells This material is partly composed of maternal plasma proteins leaking out from the intervillous space Fibrinogen which is most easily precipitated is the major constituent Some of the fibrinogen is converted into fibrin Another component of the intercellular material probably is contributed at the same time by secretory activity of the cells which seems to be more highly developed in the cells of the islands than in those of the columns Regressive changes take place in the cells of the islands and some of the cells disintegrate cellular debris contributes to the intercellular substance With increasing gestational age proliferative activity of the cytotrophoblast decreases and finally ceases completely and no further formation of islands takes place The fate of the cell islands of the immature placenta is uncertain and complete disintegration and disappearance is a possibility The described cell islands in the mature placenta seem to be cross sections of the placental septa and not isolated cell islands They may however possibly be formed in part from the islands of the immature placentas that have attached to the septa

SUMMARY

Studies by light and electron microscopy of the normal human placenta indicate that the cell islands in the immature stages are developed from the cytotrophoblastic cell columns and remain attached to those The described cell islands in the mature placenta seem to be cross sections of the placental septa

The cells of the islands at all gestational stages and the cells of the cytotrophoblastic cell columns are found to possess well developed Golgi complex and signs of secretory activity related to these structures

The intercellular materials are composed of granular masses fibrillar fibrin glycogen like granules and cellular debris

The granular masses probably represent a mixture of secretory products of the cells and material derived from the maternal blood The glycogen like granules probably originate from the cells

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HISTOLOGICAL AND HISTOCHEMICAL STUDY OF THE EXTRACELLULAR DEPOSITS IN THE NORMAL HUMAN PLACENTA

By

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Light electron microscopic and immunohistochemical studies of the normal human placenta showed that the deposits on the syncytium of the villi and of the chorionic plate (Langhans layer) as well as the deposits covering the basal plate against the intervillous space (Rohr s stria) are thrombi derived from the maternal blood (13 14 18) The thrombi are originally platelet thrombi which undergo secondary changes including accumulation of fibrin Gradually the thrombi transform into a material suggested to represent aged fibrin (18) In the present study histological and histochemical methods are applied to the normal human placenta for a further characterization of the transformed thrombi One problem is whether any methods or combination of methods are specific for aged fibrin Since the deposits have been suggested partly to represent amyloid (8) they will also be studied by methods specific for the demonstration of this material

Nitabuch s layer the deposit between foetal and maternal cellular elements in the basal plate was assumed to be a zone of demarcation composed of precipitated maternal plasma proteins and cellular debris (13) Whether this material gives reactions similar to that of aged fibrin and whether other substances are important components will be investigated One possibility is that Nitabuch s layer represents remnants of an implantation haematoma If so one would expect iron and haemoglobin derivatives to be present

The intercellular deposits of the cell islands the cytotrophoblastic cell columns and the cytotrophoblastic shell were found to have certain characteristics in common with Nitabuch s layer although secretion products of the cells are most likely admixed (15) This study tries to shed further light on the nature and genesis of these intercellular deposits

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The interstitial ground substance of the decidua differs in composition from the deposits in the placenta (9 13, 21 25) Histological and histochemical staining methods will be applied to the decidua to confirm previous observations and to examine whether material similar to that of Nitabuch s layer may penetrate into the maternal tissue

MATERIAL AND METHODS

Thirty three mature and 26 immature placentas were examined The immature placentas were obtained by therapeutic abortions in women 9 to 24 weeks pregnant (menstrual age) They had no somatic disorders and the pregnancies were interrupted on psychiatric indications Immediately after delivery or removal the placentas were gently rinsed in 0 9 per cent NaCl Specimens were taken from the central and peripheral parts and fixed in buffered formaldehyde solution (20/100) Some of the specimens were fixed for 24 hours in Zenker s fluid for PTAH staining Helly s solution for Lendrum s MSB staining and in Carnoy s fluid at 4 C for the rosindole reaction Paraffin sections were stained with

1 Haematoxylin azo phloxine (HP)
2 Staining reactions for fibrin Lendrum s Martius scarlet blue method (MSB) (10) Lendrum s Masson 44/41 method (44/41) (10) Mallory s phosphotungstic acid haematoxylin method (PTAH) (12) Heidenhain s azan stain (Azan) (20)
3 Staining reaction for tryptophan (amino acid present in plasma proteins especially in fibrinogen and its derivatives (20)) Rosindole reaction (6) counterstained with nuclear fast red
4 Staining reaction for glycogen and glycoproteins McManus periodic acid Schiff technique before and after diastase (PAS PAS diastase) (7 19)
5 Staining reactions for acid mucopolysaccharides Toluidine blue method (11) Alcian blue method (7)
6 Staining reactions for amyloid Modified Congo red method (11) examined by polarized light for positive birefringence Thioflavine T method (23)
7 Staining reactions for connective tissue Van Gieson s collagen stain (19) Verhoeff s elastic stain (19) Gomori s stain for reticulin fibres (19)
8 Staining reaction for ferric iron Turnbull method (19)
9 Staining reaction for haemoglobin Lepehne s method (2?)

For photography green or blue filter were used to increase contrast
A Leitz Orthomat microscope with UG 1 and BG-38 filters was used for fluorescence microscopy of Thioflavine T stained sections Agfa Lopan IFF film (15 Din) was used for the photographs

RESULTS

The results are summarized in Table 1

In most cases the findings were similar in placentas of 8 weeks pregnancy and in those in later stages although quantitative differences existed Unless otherwise stated the following description applies to all specimens examined

All deposits exhibited a red colour with HP stain

The Masson 44/41 method is supposed to distinguish between recent (red colour) and old (dark blue colour) fibrin (10) Such a differentiation did not seem to be valid for deposits in the placenta

Fluorescence microscopy of unstained sections showed a very weak autofluorescence of all deposits

TABLE 1

Staining Results of Deposits in Different Localizations

	Deposits on the syncytium		Rohr's stria Nitabuch's layer	Intercellular deposits of cell islands cell columns and shell	Interstitial ground substance of the decidua
	Immature stages	Mature stage			
Azan	+	+−	+	+−	−
44/41	+	+−	+	+−	−
PTAH	+	+−	+	+−	−
MSB	+	+−	+	+−	−
Rosindole	+	+	+	+	−
PAS	+	+	+	+	+
PAS/diastase	+	+	+	+	+
Toluidine blue	−	−	−	−	+
Alcian blue	−	−	−	−	+
Congo red birefringence	−	−	−	−	−
Thioflavine T	+	+	+	+−	+
Van Gieson	−	−	−	−	−
Verhoeff	−	−	−	−	−
Gomori	−	−	−	−	+
Turnbull	−	−	−	−	−
Lepehne	−	−	−	−	−

+− is used when only limited areas give a positive stain

Deposits on the Syncytium (Intervillous Deposits, Langhans Layer) and Rohr's Stria

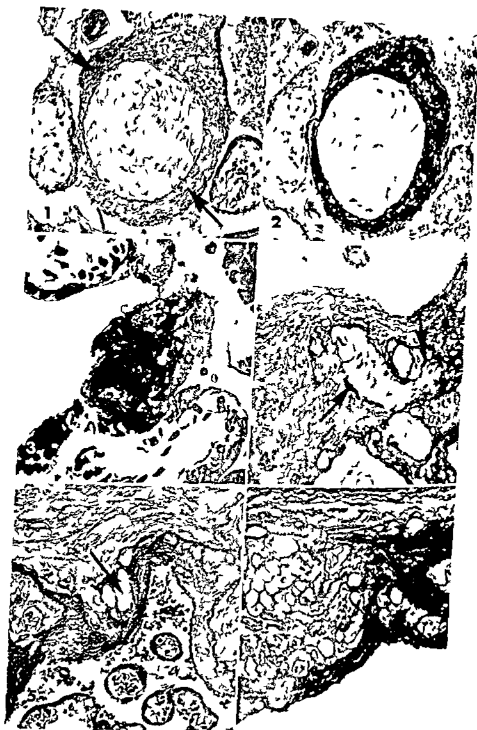
Generally the staining reactions for fibrin differed in the immature and the mature placenta In early stages almost all deposits not composed of platelets exhibited a positive stain throughout (i e red with MSB red or dark blue with 44/41 bluish black with PTAH red with Azan) (Figs 1 and 2)

In the mature placenta recent small deposits not predominantly composed of retained platelets also gave a positive stain throughout (Fig 3) The large intervillous deposits and the continuous Langhans layer often exhibited an extensive *positive staining* reaction with MSB (Figs 4 and 5) whereas the other staining reactions for fibrin (44/41 PTAH Azan) were only occasionally positive *throughout* the masses More often 44/41 PTAH and Azan gave a positive stain only in the peripheral part corresponding to areas of distinct fibrillary structures The central region on the other hand was more hyaline and tended to give a negative stain both with 44/41 (blue) PTAH (brownish red) (Fig 6) and Azan (blue)

In all placentas investigated Rohr's stria gave a positive fibrin stain

By the rosindole reaction a blue (*positive*) colour was obtained throughout all deposits (Fig 7)

PAS gave a moderate positive reaction not influenced by diastase Judged by the negative reaction by toluidine blue and Alcian blue


1
2
3
5

acid mucopolysaccharides were not present Toluidine blue however often gave a greenish colour so called negative metachromasia (1)

Congo red gave patchy positive stain of the deposits but these areas showed negative birefringence by polarized light Thioflavine T revealed pronounced fluorescence (Figs 8 and 17) although less than in amyloid

Nitabuch s Layer

The staining reactions for fibrin were positive throughout (Figs 9 and 10) at all stages of pregnancy

All other staining reactions were the same as those of the intervillous deposits Langhans layer and Rohr s stria (Table 1)

Argyrophilic fibres were demonstrated in Nitabuch s layer close to decidua (Fig 18)

Intercellular Deposits of the Cell Islands the Cytotrophoblastic Cell Columns and Shell

All staining reactions for fibrin were negative in the deposits of the cell columns of the immature placenta except for Azan which sometimes showed patches of red stain in the areas adjacent to the intervillous space

The deposits of the cell islands mostly gave a negative fibrin staining reaction early in gestation As the pregnancy advanced patches of positive stain were seen particularly in the peripheral parts (Figs 11 12 and 13) MSB seemed to give more pronounced positive stain than the other methods (Fig 13) PTAH tended to react with material of fibrous structure (Fig 11)

The findings were similar in the deposits of the cytotrophoblastic

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Figs 1 6

Fig 1 Placenta at 12 weeks Fibrous villus surrounded by a deposit giving a positive stain by MSB (arrows) Note absence of viable Langhans cells and syncytium MSB × 200

Fig 2 Placenta at 12 weeks Same as Fig 1 stained with PTAH The fibrillar structure of deposit is more apparent PTAH × 200

Fig 3 Placenta at term Deposit on syncytium of villus stained red (appears grey) with admixture of dark blue (appears black) spots Masson 44/41 × 500

Fig 4 Placenta at term Large deposit enclosing fibrous villi exhibits an extensive negative stain for fibrin Occasionally positive stain mostly located at the surface of enclosed villi and cytotrophoblastic cells (arrows) Masson 44/41 × 200

Fig 5 Placenta at term Subchorionic deposit (Langhans layer) gives a positive stain Note proliferation of cytotrophoblastic cells (arrow) MSB × 200

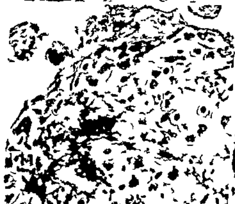
Fig 6 Placenta at term Langhans layer reveals a positive stain predominantly in peripheral parts with fibrillary structure PTAH × 200


7


V


C
N
D


N
D
10


M


12

shell and in the cell islands Positive fibrin stain was obtained near the intervillous space and Nitabuch's layer (Fig 9)

The rosindole reaction was positive with increasing intensity as pregnancy advanced (Fig 14) However the reaction was more faint than in the deposits on the syncytium, Rohr's stria and Nitabuch's layer

With the PAS technique strongly positive reaction was found both intra and extracellularly the findings were most pronounced in the cell columns (Fig 15) After diastase (Fig 16) the intracellular PAS positive reaction was abolished whereas a diffusely positive reaction remained throughout the deposits at all stages of pregnancy This reaction was stronger here than in the deposits on the syncytium Rohr's stria and Nitabuch's layer

The staining reactions for amyloid gave results similar to those described for deposits on the syncytium Rohr's stria and Nitabuch's layer although fluorescence with thioflavine T was less prominent and scattered (Fig 17)

Interstitial Ground Substance of the Decidua

The staining reactions for fibrin (Fig 10) and the rosindole reaction were negative whereas PAS showed moderate positive reaction not influenced by diastase

The presence of acid mucopolysaccharides was indicated by a purple colour in the toluidine blue preparations and a positive stain with Alcian blue

The thioflavine T method exhibited a faint fluorescence

Gomori's stain for reticular fibres was positive whereas other staining methods for connective tissue were negative

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Fig 7–12

Fig 7 Placenta at term Stem villus surrounded by *deposit stained* intense blue (appears black) Rosindole × [illegible]00

Fig 8 Placenta at 12 weeks. Fibrous villus (V) surrounded by deposit exhibiting fluorescence Thioflavine T × 400

Fig 9 Placenta at 24 weeks Basal plate *with* cytotrophoblastic shell (C) Nitabuch's layer (N) and decidua (D) Nitabuch's layer stained as fibrin Some positive stain in the intercellular *substance* of cytotrophoblastic shell and of decidua adjacent to Nitabuch's layer *Rohr's* stria is missing in this area Masson 44/41 × [illegible]00

Fig 10 Placenta at 17 weeks Nitabuch's layer (N) with a positive MSB stain The interstitial substance of decidua (D) *shows* positive stain only adjacent to Nitabuch's layer MSB × 500

Fig 11 Placenta at 14 weeks Cell island with traces of fibrillar bluish black (appears black) material near the surface PTAH × [illegible]00

Fig 12 Placenta at [illegible]4 weeks Cell island Fibrillar dark blue (appears black) material at the surface Masson 44/41 × [illegible]00


13


14




16
D


17
C


V
D
18

DISCUSSION

1 *Deposits on the syncytium (intervillous deposits, Langhans layer) and Rohr s stria* The present study shows that deposits in the immature placenta gave positive staining reactions both by histological (MSB 44/41 PTAH Azan) (10 12 22) and histochemical methods (Rosindole reaction PAS/diastase) (6 19) for the demonstration of fibrin These findings are consistent with our previous observations that the deposits are thrombi (13 18)

The large intervillous deposits and Langhans layer especially found in the mature placenta frequently gave a positive stain for fibrin in the superficial areas adjacent to the intervillous space whereas the deeper parts gave a negative staining reaction However in a previous paper (14) it was shown that with anti human fibrin/fibrinogen labelled with FITC the material gave a positive reaction throughout i e also in the deeper parts Ultrastructurally the deeper parts of the deposits consisted of granular masses which merged with fibrin strands towards the surface A few more or less preserved platelets were located in lacunae within the granular masses Therefore the granular masses were considered to represent "aged" fibrin in unresolved thrombi (18) That aged fibrin often gives negative stain with specific fibrin methods is in agreement with *Lendrum et al* (10) At the surface the positive material most likely represents more recently deposited fibrin since the thrombosis is going on continuously or repeatedly during the entire pregnancy (18)

The rosindole reaction (6) gave a positive stain throughout the de posits This method is therefore *not suited for distinction* between recent and aged fibrin and cannot be *considered* specific for the latter form

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Figs 13 18

Fig 13 Placenta at term Cell island Material stained as fibrin at the surface and in patches in central part MSB × 200

Fig 14 Placenta at 12 weeks *Cell island* Intercellular deposit stained blue (ap pears dark grey) Rosindole × 200

Fig 15 Placenta at 8 weeks Cell column Strong PAS positive reaction of both extra and intracellular (arrows) material PAS × 200

Fig 16 Placenta at 8 weeks Same spe imen as Fig 15 treated with saliva before staining PAS positive reaction of intercellular material (arrows) PAS/ diastase × 200

Fig 17 Placenta at term Fluorescence of deposits around stem villus (large arrow) and at the periphery of cell island (*CI*) Patches of fluorescent material (small arrows) in the intercellular deposit of the cell island Thioflavine T × 100

Fig 18 Placenta at *term* Basal plate with argyrophilic fibres surrounding decidual cells (D) No argyrophilic materials in foetal *part of* basal plate V villi Gomori s stain × 200

The patchy positive reaction with Congo red and the fluorescence with thioflavine T method may suggest the presence of amyloid *Horn & Horalek* (8) also found that the superficial areas of the deposits gave a positive stain with Congo red Ultrastructurally however no amyloid fibrils are found within the deposits (16) The Congo red method frequently gives false positive reactions and a negative birefringence when examined by polarized light makes the diagnosis of amyloid doubtful (4) The thioflavine T method was originally supposed to be specific for amyloid (23), but can also demonstrate fibrin (21) as well as other substances (2 3 21) The positively staining areas observed in this study may therefore well be due to the presence of fibrin or derivatives of fibrin

2 *Nitabuch s layer* is not in immediate contact with the intervillous space and is therefore not of thrombogenic origin Nevertheless the histological methods for fibrin (MSB 44/41 PTAH Azan) and the rosindole reaction were positive throughout the layer at all stages of gestation Immunohistochemically fibrin/fibrinogen fluorescence is seen throughout the layer (14) Ultrastructurally Nitabuch s layer consists mostly of uncharacteristic granular masses although cell debris and fibrin threads are found (13) The ultrastructural granular masses may represent precipitated fibrinogen aged fibrin or both originating from the maternal plasma (13 14) That the entire Nitabuch s layer gave positive reactions for fibrin in contrast to the central areas of the large intervillous deposits and Langhans layer may suggest that the ultrastructurally granular masses in Nitabuch s layer rather represent precipitated fibrinogen than aged fibrin The fact that the layer is stained as fibrin does not speak against this hypothesis since *Moe & Abildgaard* (17) showed that under certain circumstances fibrinogen also gives a positive reaction with several of the methods used in this study

The absence of iron and haemoglobin does not support the theory that Nitabuch s layer represents remnants of an implantation haematoma

3 *The intercellular deposits of the cell islands the cytotrophoblastic cell columns and the cytotrophoblastic shell* The cell islands the cytotrophoblastic cell columns and shell are collections of cells partly bounded by the intervillous space further the cells possess secretory activity (13 15) The histological staining reactions for fibrin in the deposits of these structures were usually negative early in gestation With advancing gestational age however the intercellular deposits became positive to an increasing extent particularly in regions near the intervillous space The positive stain could be due to an increasing accumulation of fibrinogen partly converted into fibrin MSB tended to stain the deposit more extensively than the other methods did this may mean that the MSB method is a more sensitive method for fibrin or fibrinogen Another possibility is that MSB stains substances other than

fibrin or fibrinogen thus in vitro this method is shown to stain macroglobulin as well (17)

Ultrastructurally the intercellular deposits were composed of cell debris uncharacteristic granular masses and traces of fibrin especially in the periphery near the intervillous space (13 15) Immunohistochemically fibrin or fibrinogen was found diffusely located throughout the masses whereas albumin was more irregularly distributed (14) It was concluded that the intercellular deposits consist of material derived from the maternal blood and from the cells in the tissue (13 14 15)

The rosindole reaction which was positive throughout the intercellular deposits may reflect the presence of fibrin or fibrinogen If so the rosindole reaction is more sensitive than the other methods for the demonstration of fibrin fibrinogen or derivatives However the positive rosindole reaction may partly be due to the presence of other substances with a high content of tryptophan

The intra and extracellular PAS positive material digested by diastase probably represents glycogen (5) this is in agreement with the finding of glycogen like granules by electron microscopy (15) After diastase the intercellular deposits revealed a diffuse PAS positive reaction throughout at all stages of pregnancy i e before the histological staining reactions for fibrin were positive The reaction was stronger than in all other deposits and may therefore represent cellular secretion products of glycoprotein nature

Amyloid was not found either with the Congo red method or ultrastructurally (15) The fluorescence with thioflavine T may therefore be unspecific attributed to fibrin fibrinogen or other substances

The absence of iron by Turnbull s method is contrary to the findings by *Dempsey & Wislocki* (5) who found a dense concentration of iron in the intercellular material associated with the cytotrophoblast

On the basis of present and previous (13 14 15) observations one may conclude that the intercellular deposits of the cell islands the cytotrophoblastic cell columns and shell are partly composed of maternal plasma proteins especially fibrinogen with its derivatives partly of other substances of glycoprotein nature probably a secretion product of the cells

4 *The interstitial ground substance of the decidua* behaved in a manner quite different from that of the deposits on the foetal side

All staining reactions for fibrin were negative

Argyrophilic fibres and acid mucopolysaccharides were present in the decidua and this fact is an important feature for distinguishing between maternal and foetal elements (13 24 25)

SUMMARY

Deposits in the normal human placenta from 8 weeks gestation up to term were examined by staining reactions for fibrin (Lendrum s MSB

and Masson 44/41 methods PTAH Heidenhain s azan) the rosindole reaction for indole derivatives periodic acid Schiff technique before and after diastase staining reactions for acid mucopolysaccharides (toluidine blue Alcian blue) amyloid (Congo red birefringence thioflavine T) connective tissue (van Gieson s Verhoeff s Gomori s stains) iron (Turnbull s stain) and haemoglobin (Lepehne s stain)

The deposits on the syncytium (intervillous deposits Langhans layer) Rohr s stria and Nitabuch s layer mostly stained like fibrin or fibrinogen with the histological staining reactions for fibrin the rosindole reaction and PAS/diastase none of the methods used seemed to be specific for aged fibrin The other staining reactions were negative except for thioflavine T which seems to be an unspecific staining of fibrin fibrinogen or their derivatives since there was no other evidence suggestive of the presence of amyloid

The intercellular deposits of the cell islands the cytotrophoblastic cell columns and shell gave a positive stain with the histological methods for fibrin in more advanced gestational stages mostly located to areas near the intervillous space indicating the presence of fibrinogen or its derivatives The PAS technique showed the presence of glycogen and a substance of glycoprotein nature which may partly represent a secretion product of the cells

The interstitial ground substance of the decidua differed from the deposits on the foetal side as the staining reactions for fibrin in decidua were negative and those for acid mucopolysaccharides and argyrophilic fibres were positive

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REVERSIBLE AND IRREVERSIBLE LDH ISOENZYMATIC ALTERATIONS IN MALIGNANT AND NON MALIGNANT MURINE TISSUES DURING CULTIVATION

A Correlation between Tumour Morphology and Isoenzyme Structure

By

E LANGVAD

Received 9 8 69

Cells of the mammalian organism exhibit a wide array of morphological and functional characteristics which represent various facets of cellular differentiation Differentiation may be considered as a process of phenotypic repression due to a continuous chain of restrictions being enforced upon the genetic code derived from the fertilized ovum As a result of this process structural metabolic and antigenic differences gradually develop between various organs and tissues of the individual during its embryonic and early life

As a manifestation of differentiation the existance of a number of isoenzymes has attracted the interest of numerous workers during recent years Thus the glycolytic enzyme lactate dehydrogenase (LDH) has been found to appear in five molecular variants or isoenzymes Each isoenzyme is a tetramer being composed of four polypeptide chains which may be one of two types called sub unit M and sub-unit H The synthesis of each sub unit is controlled by separate genes By random association of sub units the five isoenzymes represent the five possible tetrameric combinations ranging from LDH_V (M_4) to LDH_I (H_4) the hybrid enzymes being LDH_{IV} (M_3H_1) LDH_{III} (M_2H_2) and LDH_{II} (M_1H_3) Distinct catalytical physical and immunological properties are conferred on the tetramers according to the relative representation of each sub unit Thus the substrate affinities of the five isoenzymes vary in such a way that LDH_I (H_4) is inhibited at pyruvate concentrations where the function of LDH_V (M_4) is unaffected (*Apella & Markert* 1961 *Cahn et al* 1962 *Dawson et al* 1964 *Kaplan & Ciotti* 1961 *Markert & Apella* 1961 *Markert & Møller* 1959 *Plagemann et al* 1960a *Plagemann et al* 1960b *Vesell & Bearn* 1957 *Vesell & Bearn* 1961

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Wieland & Pfleiderer 1961) Electrophoretically the isoenzymes exhibit decreasing mobilities according to the relative representation of sub unit M. The LDH isoenzyme patterns of the mature organism are stable and specific for cells and organs within the same species. During foetal development characteristic sequential alterations of the LDH isoenzyme patterns occur consisting of a redistribution of the total LDH activity among the five isoenzymes (*Philip & Vesell* 1962, *Vesell et al.* 1962).

While in the intact organism all traits of differentiation are main tained within narrow limits, removal of a cell from its micro environ ment leads to rapid loss of differentiation evidenced at both the mor phological and molecular level. This is clearly seen in tissue culture in which alterations of the LDH isoenzyme pattern occur (*Philip & Vesell* 1962, *Vesell et al.* 1962). In long term cultures derived from different non malignant tissues a uniform isoenzyme pattern dominated by sub unit M will eventually result. This development has been shown to be accompanied by an increased glycolytic capacity (*Paul & Pearson* 1957a, b, *Briand* 1969).

In most malignant tissues a similar shift towards LDH isoenzyme patterns dominated by sub unit M is a common feature. This is in agreement with the high glycolytic capacity of neoplastic tissue.

In the present study the LDH isoenzyme pattern has been used as an indicator of differentiation. The isoenzymatic structure of normal and malignant murine tissues has been studied before and during cultivation *in vitro*. Attempts have been made to correlate the iso enzymatic changes occurring *in vitro* with the process of spontaneous malignant transformation and with the morphological differentiation of tumours produced in susceptible animals by reinoculation of cul tured malignant tissues.

MATERIAL AND METHODS

Normal and malignant tissues derived from inbred ST/a, C3H or DBA/2 mice were explanted to Fibiger flasks using the following technique. The tissues were minced with fine scissors and spread on the bottom of the empty culture flasks with sterile stainless spatula. The flasks were then turned upside down and 4 ml of culture medium Fib 14B (*Biesele et al.* 1958) were added. After gassing with 5 per cent CO_2 in 20 per cent O_2 the flasks were closed and placed in an incubator at 37° C still upside down. After 20 minutes of incubation when the explants adhered firmly to the bottom the flasks were turned round carefully so that the tissues were covered with the medium. Using this procedure the explants remained adhered to the bottom without the use of plasma clots for more than two weeks provided that the flasks were not shaken.

Tissue for reinoculation or for isoenzymatic assay was harvested after varying periods of cultivation. Prior to homogenization tissue for enzymatic studies was washed repeatedly in order to remove cultivation medium and erythrocytes, first in physiological saline with 0.75 ml of EDTA added per 1000 ml and finally in destilled water in order to haemolyse remaining erythrocytes. Homogenization was carried out using a glass teflon homogenizer. After centrifugation at 1700 × g for 15 min utes 30 µl samples of the supernatant were used for the disc electrophoretic separa tion of the isoenzymes. All procedures were carried out at temperatures below 5° C.

The disc electrophoretic procedures as well as the visualization and quantitation of the enzyme activities were as previously described (*Langvad* 1968 a, b). Using

spacer and separation gels of 3 and 7 5 per cent acrylamide at pH 6 7 and 8 8 respectively and a glycine Tris/(hydroxymethyl)/aminomethane buffer (Si ma St Louis Mo USA) at pH 8 3 five LDH isoenzymes were resolved from murine tissue homogenates Under identical conditions only four LDH isoenzymes are resolved from human tissue homogenates since here LDH_5 does not enter the separation gel

Biopsies from tumours were divided one half being used for enzymati studies and one for histological examination The tissues were fixed in 5 per cent neutral formalin Paraffin sections at 3 μ were stained with haematoxylin and eosin

The C3H L, C3H M and C3H E cells were established in 1964 the Lu 310 Lu 3120 Lu 3100°0 AcP and STR in 1965 The STL cells ori inate from 1966 while C3H P C3H SB and C3H–TVM were established in 1968

The antilymphocytic sera used in this study were kindly supplied by dr Balzi kowsl i They were produced by the immunization of rabbits with mouse spleen or thymus Adult animals were treated with 0 1 ml of ALS subcutaneously for 10 con secutive days starting one day prior to transplantation of the cells to be tested for tumourigenecity

The sub unit M mediated LDH activity expressed as per cent of the total LDH activity was calculated on basis of the known tetrameric composition of the five isoenzymes and was used as an indicator of the isoenzymatic structure

RESULTS

The LDH isoenzymatic structure of normal murine tissues is shown in Table 1 In Table 2 induced or spontaneous murine tumours are listed in order of decreasing sub unit *M mediated LDH activity* It appears that all tumours with M activities of less than 90 per cent were highly or fairly well differentiated Typically sarcomas and anaplastic car cinomas exhibited activites above 91 per cent

TABLE 1

Sub Unit M Mediated LDH Activity in Normal Murine Tissues as Per Cent of Total Activity

Tissue	per cent sub unit M activity
Cerebrum	31 0
Ovary	40 8
Whole kidney	43 8
Heart	44 4
Adrenal	62 0
Glandular stomach	66 7
Uterus	70 0
Post pubertal testis	70 9
Lung	71 6
Mamma	73 5
Non glandular stomach	74 3
Striated muscle	77 6
Subcutaneous tissue	81 5
Oesophagus	81 9
Colon	82 4
Small intestine	8? 6
Spleen	90 2
Pancreas	90 5
Liver	91 5
Full thickness skin	92 5

The C3H L M and E cells were explanted by *J Kieler* the Lu cells by *P Briand* while the remaining cell lines were established by the author

TABLE 2

Sub Unit M Mediated LDH Activity as Per Cent of total Activity in Murine Tumour Tissues

Tissue or origin	Type	per cent sub unit M activity	Induction
Bronchus	anaplastic carcinoma	97 9	Urethane
Retroperitoneal tissue	pleomorphic sarcoma	97 8	Urethane
Epidermis	anaplastic carcinoma	97 7	DMBA
Liver	hemangioendothelioma	97 7	Urethane
Subcutaneous tissue	fibrosarcoma	97 5	Urethane
Myometrium	leiomyosarcoma	97 0	Iron Dextran
Spleen tissue	undifferentiated sarcoma	96 7	malignant transformation in vitro
Lung tissue	undifferentiated sarcoma	96 3	malignant transformation in vitro
Spleen tissue	undifferentiated sarcoma	96 1	malignant transformation in vitro
Whole embryo	undifferentiated sarcoma	94 8	malignant transformation in vitro
Spleen tissue	undifferentiated sarcoma	94 4	malignant transformation in vitro
Mamma	AQ carcino sarcoma	94 4	Spontaneous
Liver	hepatoma	93 9	Urethane
Bronchus	anaplastic carcinoma	92 4	Urethane
Lung tissue	undifferentiated sarcoma	92 2	malignant transformation in vitro
Bronchus	anaplastic carcinoma	91 7	Urethane
Mamma	highly differentiated adenocarc	89 4	Spontaneous
Mamma	highly differentiated adenocarc	89 2	Spontaneous
Epidermis	parakeratinising epidermoid carc	89 2	DMBA
Mamma	highly differentiated adenocarc	86 9	Spontaneous
Mamma	highly differentiated adenocarc	86 7	Spontaneous
Liver	cholangioma	85 7	Urethane
	chondroma	85 2	Spontaneous
Mamma	adenocarcinoma	82 4	Methylcholanthrene
Lung	highly differentiated adenocarc	82 0	Urethane
Adrenal	neuroblastoma	81 1	DMBA
Liver	cholangioma	74 1	Urethane

The history of this tumour has been reported by *Ringsted* (1957)

TABLE 3

Sub Unit M Mediated LDH Activity as Per Cent of Total Activity in Primary Explants of Non Malignant Murine Tissues

Tissue	Strain	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14 days
Lung	C3H	71 6			84 8		86 5		89 7							
Lung	ST/a	76 3		81 0		88 4			93 9					94 6		95 8
Lung	ST/a	75 7	75 1	80 2	79 9	86 6										
Lung	ST/a	69 0	7? 7	76 5	83 0											
Lung	ST/a	69 6	70 4	76 6		83 3										
Lung	ST/a	69 7	76 0		85 6											
Spleen	C3H	90 [illegible]		88 2		87 3		86 1								
Kidney	C3H	43 8		36 1	37 7	50 0		48 0	50 3							

TABLE 4

Development of M Mediated LDH Activity as Per Cent of Total Activity in Long Term Cultures of Murine Tissues in the Process of Spontaneous Malignization Values Obtained from Cultures after Malignant Transformation are in Italics

Tissue of origin	Designation	Duration of cultivation (weeks)												
		0	3	8	14	17	25	28	30	32	36	52	124	132
Lung	STL	76 3			92 3							*94 0*		96 6
Lung	C3H P	71 6	94 6	90 3		89 3		90 6		*95 7*				
Spleen	C3H SB	90 2		91 4		92 4		91 7	*94 0*					
Kidney	STR	43 8					*86 9*						*97 1*	
Striated muscle	C3H TVM	77 [illegible]		85 0					83 7		87 1			

* Value after loss of tumourigenecity

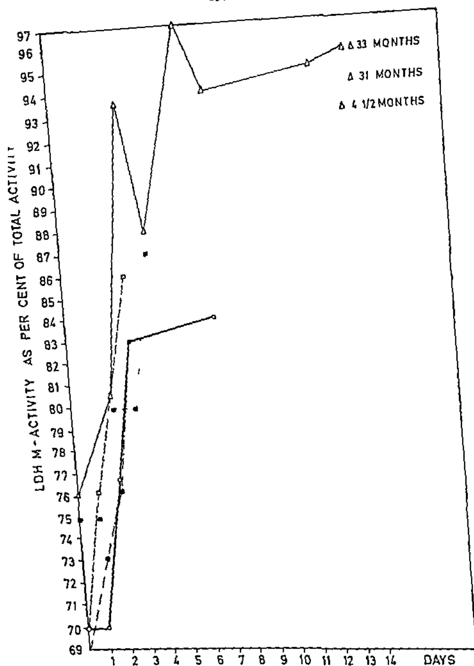


Fig 1

De elopment of LDH M activity in primary explants of normal murine lung tissue

Table 3 illustrates the development LDH M activities in primary *explants of non malignant murine tissues* While in explants of spleen and kidney an initial decrease of sub unit M activity was *noted a rapid* increase was found in all explanted lung tissues It appears from *Table 3* and Fig 1 that after 2–3 days of cultivation normal lung tissues *exhibited an LDH* isoenzymatic composition similar to that of a highly differentiated adenocarcinoma of the lung (Table 2) while 6–7 days of cultivation led to an enzyme pattern comparable to that of an anaplastic carcinoma of the lung in the same strain of mice

The LDH M activity in long term cultures of originally non malignant tissues undergoing 'spontaneous malignant transformation is shown in Table 4 It appears that following the rapid initial changes the enzyme pattern stabilized at a level above the original Finally when malignant transformation occurred a concommitant secondary *rise of the sub unit M mediated LDH activity was noted*

TABLE 5

LDH M Activity as Per Cent of Total LDH Activity of in vitro Transformed Murine Cells and the Corresponding Transplant Tumours

Strain	Tissue of origin	Cell line	Per cent M activity	
			Culture	Transplant tumour
ST/a	Lung	Lu 3105	90.1	98.1
ST/a	Lung	Lu 3120	93.7	95.3
ST/a	Lung	*Lu 310020*	*91.3*	90.1
C3H	Lung	C3H L	96.7	[illegible]
C3H	Lung	C3H P	95.7	96.3
C3H	Spleen	C3H M	95.6	[illegible]
C3H	Spleen	C3H SA	98.7	94.4
C3H	Spleen	C3H SB	94.0	96.7
C3H	Whole Fetus	C3H-E	94.4	94.8

The LDH structure of *in vitro* transformed cell lines and their corresponding tumours are shown in Table 5 Histologically *these tumours* were all undifferentiated sarcomas with M activities exceeding 90 per cent Only slight modifications of the LDH structure occurred *when* on reinoculation the transformed cells produced a tumour On explantation *in vitro* of malignant tissues the LDH isoenzymatic *pattern* exhibited an instability similar to that found for non malignant tissue It appears from Table 6 that enzymatically the general *effect of cultivation* was that of an increase of M mediated LDH activity Although in some cases an initial decrease was noted prolonged *cultivation resulted in* a significant increase of M activity

The results of retransplantation of cultivated tumour *tissue are presented* in Table 7 It appears that the enzymatic changes induced in tumour tissues during short time cultivation were *largely reversible* when the tissue was reintroduced into the environment of the intact

TABLE 6

LDH M Activity as Per Cent of Total LDH Activity in Primary Explants of Malignant Murine Tumours

Tumour	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14 days
Adenocarcinoma pulm	92 5	89 4	88 9	94 0	91 3		96 0	95 2			90 5				
Cholangioma	85 7			91 0			93 0			93 5					
Chondroma	85 0		81 1				75 0			78 7					89 5
Mammary carc	86 0		83 0												
Mammary carc	89 2				88 7										
Mammary carc	81 0				83 9										88 9
Mammary carc	82 4														94 7
Mammary carc	83 5							91 5							

TABLE 7

Sub Unit M Mediated LDH Activity in Cultured Murine Tumour Tissues and Tumours Following Retransplantation

Original tumour	Age of culture (days)	Tumourigenecity	Per cent LDH M activity cultures	Per cent LDH M activity tumours	Histological differentiation
Mammary carcinoma	0	+		81 0	high
	4	+	83 9	91 3	high
Mammary carcinoma	0	+		89 2	high
	4	+	88 7	87 4	high
Mammary carcinoma	0	+		8[illegible] 4	medium
	14	+	94 7	84 4	medium
Mammary carcinoma	0	+		86 0	high
	2	+	83 0	83 9	medium
Epidermoid carcinoma of skin	0	+		89 2	high
	21	+	96 0	86 5	low
	6[illegible]	+	92 2	92 4	low
Adenocarcinoma of lung	0	+		82 0	high
	[illegible]	+	98 0	89 4	medium
	195	+		91 7	low
	395	+	95 1	92 4	low
	950	—	96 0		
Chondroma	0	+		85 0	high
	2	+	81 1	84 4	high
	6	+	75 0	86 0	high
	9	+	78 7	87 0	high
	14	—	89 5		
	4[illegible]	—	93 0		
	60	—	97 0		

organism It also appears that in most of the tumours resulting from reinoculation of tumour tissue cultured for a short period of time the histological structure of the original tumour was more or less reproduced After prolonged periods of cultivation the tumourigenecity was retained in many cases but the ability to produce differentiated tumours was lost completely and the LDH isoenzyme pattern failed to revert from that established in tissue culture

In other cases the tumourigenecity seemed to be entirely lost both in 6 days old mice non conditioned adult mice and in adult mice treated with antilymphocytic serum (Table 8) Thus it may be seen from Table 7 that the chondroma cultivated for 2 6 and 9 days was fully capable of reproducing the original tumour morphology as well as the initial isoenzymatic structure Between the 9th and 14th day of cultivation the tumourigenecity however was lost while at the same time the M mediated LDH activity exhibited a sharp increase Concomitantly *the chondrocytes were noted to dwindle and eventually disappear* from the cultures which were overgrown by stromal cells

At 33 months the cell line AcP derived from the pulmonar adenocarcinoma (Table 6) which on reinoculation had regularly produced anaplastic carcinomas had lost its tumourigenecity while the isoenzymatic pattern remained the same with an M activity above 96 per cent Similarly the cell line STL which had undergone malignant transformation *in vitro* eventually lost its tumourigenecity while at the *same* time the LDH M activity exhibited a further increase (Table 4)

DISCUSSION

It has been maintained that the *in vitro* conditions favour the *selection* of a ubiquitous cell type Thus *Sato and colleagues* (1960) found that rat liver cells propagated *in vitro* possessed antigens common to the antigens found in cultured cells derived from kidney while they lacked certain antigens found in liver cells *in vivo* A *selection in vitro* of cells common to different organs certainly would lead towards the establishment of an LDH *isoenzyme* pattern common for cultures derived from different tissues

The theory of selection is countered by a *theory* of dedifferentiation suggesting that the cellular and biochemical interactions present *in vivo* are responsible for the maintenance of *differentiation* Therefore under *in vitro* conditions cellular differentiation is lost due to the disappearance of the complex micro *environmental* factors

The observations made *in this* study are in favour of the concept of de-differentiation The LDH isoenzymatic alterations occur with such abruptness and rapidity that a positive *selection* of cells adaptable to *in vitro* conditions offers no reasonable explanation

The finding that the isoenzymatic alterations developed during short

TABLE 8

Subcutaneous Transplantability of STL AcP and Chondroma Cell Cultures into Conditioned Adult Unconditioned Adult and 6 Day Old ST/a Mice

Cells		STL			AcP			Chondroma	
Recipient animals	6d	C A §	U A †	6d	C A	U A	6d	C A	U A
No of cells inoculated	1×10^6	25×10^6	25×10^6	1×10^6	25×10^6	25×10^6	1×10^6	25×10^6	25×10^6
No of animals	4	2	4	4	2	8	1	3	3
No of takes	0	0	0	0	0	0	0	0	0

6 days old § Conditioned adult † Unconditioned adult

time cultivation are reversible on reintroduction into the intact organ ism indicate that the enzymatic modifications are resulting from alterations of the functional state of the genes controlling the produc tion of sub unit M and sub unit H due to the withdrawal of controlling signals present *in vivo*

It is conceivable that the cellular apparatus necessary for the recep tion and interpretation of intercellular biochemical signals remains intact during short time cultivation while prolonged cultivation leads to a premanent loss of these functions which unify all cells into an organism Thus the reversible fluctuations of the molecular patterns observed in primary explants appear to be due to the loss of controlling environmental factors while the irreversible changes observed after prolonged periods of cultivation may be cellular structural changes caused by a degradation of the phenotype established through the process of differentiation

During prolonged cultivation progressive alterations occur Iso enzymatic and antigenic patterns originally specific for the cells are lost as well as the complex of abilities necessary for the participation in organized tissue formation and at the same time new properties such as new antigens may develop When eventually spontaneous malignization occurs this may be considered another step of dediffer entiation

It has been pointed out (*Cahn et al* 1962 *Dawson et al* 1964) that the cathodic LDH isoenzymes predominating in neoplastic tissues are those best suited for anaerobic metabolism Since in many fast growing tumours vascularization and oxygenation may be insufficient the iso enzymatic modifications characterizing tumour tissue would represent a metabolic rationale The isoenzymatic change *per se* however is no criterion of malignancy since it may be induced in cells which are non tumourigenic Although in the present material isoenzymatic modifica tions were found in all tumours no conclusions can be drawn as to the significance of this metabolic change in the pattern of malignant behaviour

However a clear correlation was established between the LDH iso enzyme pattern and the histological differentiation of murine tumours In well differentiated carcinomatous tissues cells exhibit a high degree of normal qualities present in the tissue of origin This is evidenced both at the morphological and the molecular level Thus carcinomas presenting a high or fair degree of differentiation registered LDH M activities of less than 90 per cent while anaplastic carcinomas ex hibited M activities above 91 per cent

Similarly the loss of differentiation found in tumours produced by originally highly differentiated tumour tissue cultivated for prolonged periods of time was accompanied by increased M activity

In cultured non malignant lung tissues LDH M activities comparable to those of anaplastic carcinomas were induced within a matter of days

while spontaneous malignization was a late event occurring after 30 weeks of cultivation or more

While malignancy as evidenced by tumour producing capacity was in all cases accompanied by an increased LDH M activity cultured tumour tissue and tumour producing cell cultures might lose tumourigenecity while retaining a high level of M activity In the case of cultivated *chondroma tissue* this was clearly due to a selection of stromal cells In the case of the cell line STL and that derived from a pulmonar adenocarcinoma both of which eventually lost their tumour producing capacity while retaining high LDH M activities no morphological evidence of selection was found Also the loss of tumourigenecity could not be explained by antigenic changes since no tumours were produced neither in 6 days old nor in adult animals conditioned with antilymphocytic sera

Thus murine neoplasia is accompanied by increased M activities but *the enzymatic change per se is no indicator of tumourigenecity*

The prolonged latency from the establishment of isoenzymatic changes *in vitro* until the occurrence of malignant transformation might indicate that the isoenzymatic changes bear no relationship to neoplasia Studies of human neoplasia however support the assumption that these isoenzymatic changes may be very early indicators of advancing carcinogenesis LDH isoenzymatic changes comparable to those characterizing neoplastic tissue are found in apparently non affected regions of tumour bearing organs (*Langvad* 1968a b) as well as in various conditions recognized as being potentially preneoplastic (*Yasin & Bergel* 1965 *Leese* 1965 *Langvad & Roed Petersen* 1969)

Thus the LDH isoenzymatic pattern of murine tissues subjected to experimental carcinogenesis *in vitro* as well as human tissues in the process of malignization exhibit *alterations preceeding the stage of* tumourigenecity and morphological malignancy The correlation of isoenzyme pattern and histological differentiation in murine *tumours* indicates that the enzymatic modifications are early signs of dedifferentiation rather than criteria of established malignant transformation

SUMMARY

The lactate dehydrogenase isoenzyme pattern of malignant and non malignant tissues explanted *in vitro* exhibit rapid changes towards preponderance of isoenzymes dominated by sub-unit M The isoenzymatic changes *in cell lines* propagated *in vitro* have been followed in the process of spontaneous malignization

The morphology of *non cultured tumours* correlated well with the isoenzyme patterns Thus sarcomas and anaplastic carcinomas all exhibited sub unit M mediated LDH activities above 91 per cent while M activities below 90 per cent were registered for well differentiated carcinomas

The initial isoenzymatic changes induced in cultivated tumour tissue proved to be reversible on reinoculation into susceptible animals. Also the original tumour morphology was reproduced in the ensuing tumours. Prolonged cultivation however resulted in irreversible isoenzymatic changes and loss of the ability to reproduce the original tumour morphology.

Explantation of non malignant lung tissue was followed by a rapid increase of LDH M activity. When in long term cultures of non malignant tissues spontaneous malignization occurred this was accompanied by a further increase of sub unit M mediated LDH activity.

The LDH isoenzymatic modifications observed during carcinogenesis appear to reflect at the molecular level the loss of differentiation observable at the morphological level in neoplastic tissue.

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COUNTING AND ASSESSING THE SIZE OF BACTERIA WITH AN AUTOMATIC PARTICLE COUNTER

By

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Received 23 xii 68

The methods now most commonly used for counting bacteria involve microscopy or culture of bacterial dilutions *e g* by spreading on solid media Under proper conditions the accuracy obtained by these methods is reasonable However both methods are very time-consuming counting by culturing takes at least 18–24 hours Other techniques such as determination by weighing or turbidimetry provides only a rough estimate of the cell number

The need for a rapid and more accurate estimation of the number of bacteria in a suspension calls for some means of automatic counting Earlier studies of automatic bacterial counting (*Kubitschek* 1956 *Lark & Lark* 1960 *Toennies et al* 1963 *Allison et al* 1962 *Harvey & Marr* 1966 *Truant et al* 1962 *Swanton et al* 1962 *Curby et al* 1963 and *Mountney & O Malley* 1966) were successful to some extent

In the present investigation the most suitable conditions for counting various types of bacteria with the electronic particle counter Celloscope 202 were studied

MATERIALS AND METHODS

Ba[illegible]a Tests were made with rods *Esch[illegible]richia coli* (SBL 945/56) *Salmonella typh[illegible]murium* (SBL 1459/65) and *Salmonella manhattan* K–1575/66 and with cocci *Strep[illegible]us faecalis* (SBL 16908/66) and *Neisseria gonorrhoeae* (SBL 19154/67) *[illegible]ing [illegible]ounting* In order to determine the number of viable *E coli* and *Salmon[illegible]lla* ba[illegible]ter[illegible]a 1 ml aliquots of 10 fold dilutions of broth cultures in phosphate buffered saline (PBS) were poured into petri dishes (90 mm diameter) containing 15 18 ml of melted agar at 45 C. The determinations were done *in duplo* The dilutions were then incubated for 18 hours at 37 C, after which the colonies were [illegible]unted and the average calculated The dilutions selected for counting were those wh[illegible]h re[illegible]ult[illegible] in 30-300 [illegible]olonies The counting of the streptococci and gonococci was [illegible]erf[illegible]rm[illegible] in a similar manner although 0 5 ml aliquots of the dilutions were [illegible]read on the surface of bl[illegible]od agar and McLeod haematin agar respectively The

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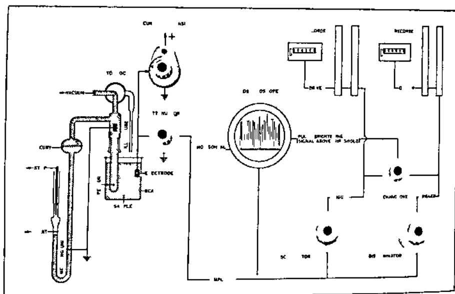


Fig 1
Principles of Celloscope 202

gonococci were derived from a culture on solid medium suspended in PBS These bacteria were incubated in a CO_2 atmosphere

Construction and use of the Celloscope The instrument was described in detail in connection with the determination of gel and sand particles i viscous solutions (*Kolos & Treiber* 1961) Therefore only a brief description will be given here

The principles of the instrument are shown in Fig 1 A fixed volume of a particle suspension in a suitable electrolyte (usually physiological saline) is sucked through a very narrow channel (the aperture) in a glass tube An electric current flows through this aperture between electrodes on each side of it

Each time a particle passes through the aperture the resistance of the circuit increases giving rise to an electric pulse the amplitude of which is proportional to the volume of the particle The pulse is then amplified displayed on an oscilloscope screen and passed through a discriminator to an electronic recorder The discriminator blocks all pulses below a preselected level Since discriminator and amplifier levels are both adjustable the instrument can be used to measure the size of particles The oscilloscope displays a picture of the size distribution which guides the discriminator setting

Apart from the two recorders built in (Fig 1) the Celloscope used in the study was slightly modified in that a third recorder was connected Since this recorder was not in direct mechanical contact with the instrument the acoustical effects which occasionally disturbed the counts at high amplification were eliminated Later models of the Celloscope consist of two separate units for this very reason

Amplification is varied either by means of a switch for different amplification factors or by means of a potentiometer which regulates the voltage between the electrodes

The settings of the instrument bear the following relationship to the size of the particle

$$d^3 = c \frac{D}{F \cdot P}$$

where d is the diameter of a sphere having the same volume as that of the particle (the equivalent sphere) c a constant which is dependent on the size of the aperture and the conductivity of the electrolyte and D F and P the numerical values for discriminator setting amplifying factor and potentiometer setting respectively

The aperture chosen for this study of bacteria was 30 microns in diameter and about 50 microns in length The aperture tube and instrument were calibrated with latex particles of 1 17 and 2 05 microns in diameter

A prerequisite for using this calibration for counting and sizing bacteria is that either their conductivity is negligible in relation to that of the suspension medium or their conductivity in the solution is known

The experiments performed in this study demonstrated that individual viable bacteria of the strains employed could be regarded as insulators when suspended in physiological saline therefore the bacterial size could be given directly as the diameter of the equivalent sphere

Precautions were taken to keep dust and electrical and acoustical disturbances at a minimum

The solutions used for the bacterial suspension were passed through membrane filtres with a pore size of 0 3 microns This filtration reduced the number of extraneous particles to 1000–1500 per 0 05 ml saline These values were acceptable as long as the concentration of bacteria was fairly high If lower concentrations were used the number of extraneous particles must be reduced even further by filtering and making all measurements in a dust free environment

The volume of suspension used for each count was 0 05 ml The time required to make the count in the instrument was about 20 seconds

Four hour broth cultures were diluted to between 1 900 and 1 1000 in 0 9 per cent saline so that the number of bacteria in 0 05 ml would lie in the range 15 000–30 000 The number of extraneous particles in an equal volume of control dilutions of broth in saline amounted to 1500 or less

RESULTS

Behaviour of bacteria in an electric field A few points has to be taken into consideration when the actual counts are made chiefly the question whether individual bacteria in physiological saline can be considered as insulators when they come into contact with the electric field of the instrument

If a particle has an appreciable conductivity in relation to the medium in which it is suspended the pulse to which it will give rise will be smaller than that caused by a completely insulating particle of the same volume This loss in pulse strength will become more marked if the conductivity of the electrolyte is reduced by dilution provided such a dilution does not influence the volume of the particle itself

The mean size of bacteria from a culture of *Escherichia coli* was determined at three different electrolyte concentrations 0 22 per cent 0 90 per cent and 4 0 per cent NaCl The Celloscope and aperture tube were calibrated with latex particles for each of these concentrations

Fig 2 shows the size distribution of bacteria at the three concentrations There is complete agreement of the mean size within the range of experimental error Therefore viable bacteria can be considered as insulators within this range of concentrations which corresponds to resistivities of 1ɔ–280 ohm cm Different findings might be expected in the case of dead bacteria depending on the degree of disintegration

The experiments also showed that the instrument was more sensitive when 4 per cent saline instead of physiological saline was used for bacterial counting However the use of 4 per cent saline also increased

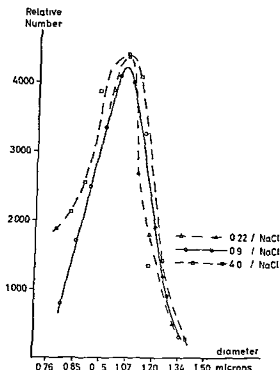


Fig 2

Size distribution of *E coli* at three different electrolytic concentrations after incubation for four hours Size expressed as the diameter of the equivalent sphere

the noise level due to the stronger current passing through the aperture The real gain therefore does not fully correspond to the increase in the sensitivity of the instrument Later experiments were performed with 0 9 per cent saline which gave wholly acceptable results

Influence of coincidence effects As the concentration of bacteria increases the instrument exhibits an increasing coincidence loss *i e* when two or more particles pass simultaneously through the "aperture channel and are recorded as one The results were corrected for this loss by using the following expression

$$N = \frac{n}{1 \; V (c \; n)}$$

where N is the corrected number n the reading on the counter for a 0 05 ml of suspension and c a constant which for the 30 micron aperture used was given as 3 10^{-6}

Fig 3 shows that counts corrected for coincidence loss nevertheless were too low when the concentration of bacteria exceeded 600 000 per ml Therefore this level was chosen as the upper limit for all experiments

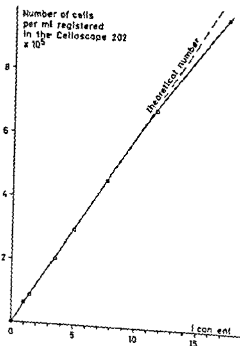


Fig 3
Accuracy of the Celloscope count at various cell concentrations
(4 hrs culture of *E coli*)

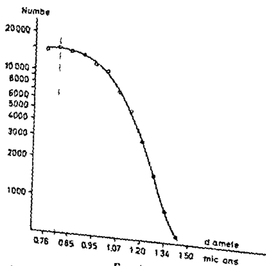


Fig 4
Cell scope count of *E coli* as a function of the instrument setting Setting expressed a the diameter of the equivalent sphere recorded (4 hrs culture)

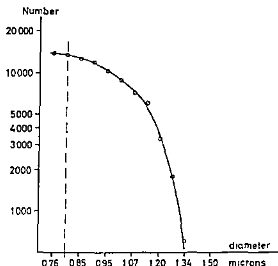


Fig 5

Celloscope count of *Salmonella typhi murium* as a function of the instrument setting Setting expressed as the diameter of the equivalent sphere recorded (4 hrs culture)

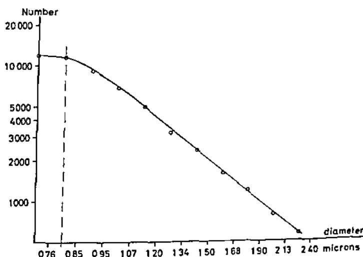


Fig 6

Celloscope count of *Streptococcus faecalis* as a function of the instrument setting Setting expressed as the diameter of the equivalent sphere recorded (4 hrs culture)

Setting of the instrument for estimating the total number of bacteria The most suitable size threshold was arrived at by repeated counts of the various cultures at gradually decreasing discriminator levels

In Figs 4 5 and 6 the counts of three strains are plotted against the discriminator level The counting levels also are indicated

In principle the setting should permit the counting of all bacteria at the same time as any disturbing factors and extraneous particles

are kept at a minimum The counting level chosen for all the strains corresponded to an equivalent sphere of 0 80 microns As will appear from the figures the maximum size of the streptococci appeared to be considerably larger than that of the other two strains This is due to the fact that several cocci arranged in chains will be recognized as one particle

When the very narrow aperture (30 microns) occasionally became blocked, the count had to be rejected and the aperture cleaned before a repeat count could be made If it was found that blockage was more than usually frequent the entire bacterial suspension was filtered through a nylon net with a mesh size of 10 microns Separate experiments demonstrated that the loss of bacteria caused by this extra filtration did not exceed [illegible] per cent of the total number at the particular concentration used

TABLE 1

Automatic Particle Counts Compared with Colony Counts on Cultures of Escherichia coli *after an Incubation Period of 3–5 Hours*

Celloscope count × 10	Plate counting × 10[4]
4.58	4.45
6.40	5.92
3.40	3.52
4.24	4.36
3 8	3.24
4.86	4.90
2.71	2.59
2.86	2.85
2.45	2.48
6.06	6.00

TABLE 2

Automatic Particle Counts Compared with Colony Counts on Cultures of Salmonella typhimurium *after an Incubation Period of 3 5 Hours*

Celloscope count × 10[8]	Plate count × 10[8]
2.20	2.23
2.00	1.94
6.36	7.00
1.90	1.95
2.13	2.35
4.80	4.65
4.30	4.36
2.14	2.23
2.20	2.20
3.20	3.10

TABLE 3

Automatic Particle Counts Compared with Colony Counts on Cultures of Streptococcus faecalis *after an Incubation Period of 4–8 Hours*

	Celloscope count × 10[9]	Plate count × 10[9]
4 hrs	1.13	0.93
	1.13	1.00
	1.33	1.30
	1.16	1.45
8 hrs	5.0	5.0

Dilution for the colony counting made 20 minutes later the Celloscope counting

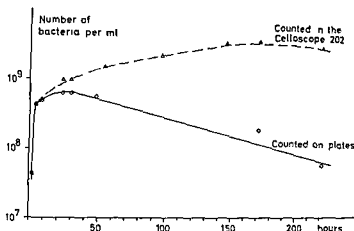


Fig 7
Comparison between the number of particles counted by the Celloscope and the number of colony forming units of *E coli* after various incubation periods.

Reproducibility Twenty independent counts of a bacterial suspension of *E coli* at the same instrument setting resulted in a standard deviation of (±) 0 53 per cent for about 20 000 bacteria

Automatic particle counts compared with colony counts on plates 3–5 hour cultures of *E coli* and *S typhimurium* were counted both in the Celloscope and by the plate method A number of comparative counts were performed also on *S faecalis* The results are given in Tables 1 2 and 3

The differences were within the experimental error The systematic error of the Celloscope itself is considered to be less than 2 per cent for the conditions of this study The differences between the results obtained in a series of counts followed a poissonian distribution The error for colony counting of *E coli* using duplicate plates was found to be in the region of (±) 4–5 per cent

The longer the period of incubation the greater the numbers of dead cells Since the electronic equipment counts all particles above a certain size irrespective of whether they are viable or dead and since the plate method of counting provides an assessment of only the number of viable cells the difference between the results of the two methods increases with increasing incubation time Fig 7 illustrates this relationship The two methods gave similar results up to about 10 hours above this level the results began to diverge

Size determination of Escherichia coli The results of size determination of a 4 hour broth culture of *Escherichia coli* are presented in Fig 2 The assessment was made by performing a series of counts beginning at the discriminator setting used for determining the total number of bacteria and then raising the level in a stepwise manner for each succeeding count so that the recorded particle volume in

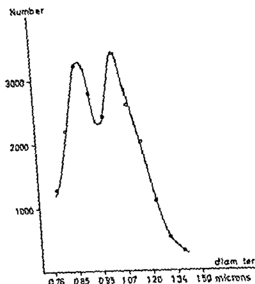


Fig 8

Size distribution of *E coli* after an incubation period of 200 hours Size expressed as the diameter of the equivalent sphere

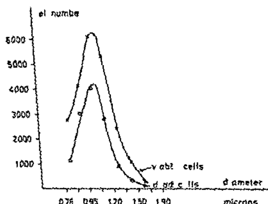


Fig 9

Comparison of the size distribution of *Salmonella typhi murium* in cultures of viable and killed organisms

creased by the factor $\sqrt[4]{2}$ at each step The mean size of *E coli* as shown by the curves in Fig 2 was approximately 1 03 microns expressed as the diameter of the equivalent sphere

The size distribution of the same culture of *E coli* after 200 hours incubation is shown in Fig 8 There are now two peaks in the curve one at 1 03 microns and another at 0 90 microns

The purity of this culture was tested by subcultures and microscopy of stained smears to see whether there had been contamination by other bacteria during incubation No contamination was demonstrable

In an attempt to test whether the two different peaks represented dead and living cells further experiments were made with cultures of *Salmonella typhi murium* in the living and the dead state The *Salmonella* bacteria were killed by the addition of one tenth volume of 0 1 M potassium cyanide

The results of the determination of the size distributions of the two cultures are shown in Fig 9

The size distribution of living or dead Salmonella cells were identical showing a mean size of 1 03 *microns*

DISCUSSION

The experiments of this study were performed chiefly on gram negative rods of the Enterobacteriaceae group which are particularly suitable for counting in an automatic particle counter because of their relatively large size and because they do not regularly appear in aggregates A number of experiments were performed also on streptococci which occurred in chains consisting of an average of 3–5 cells each

The comparison of automatic particle counting and colony counting by the plate dilution method agreed well for cultures of a few hours incubation time The good agreement between the two methods applied to bacilli as well as to chain forming cocci The latter showed that the particle counter recorded the chains as entities as is the case in colony counting

Differences between the two methods of counting were found when the cultures were incubated for more than 10 hours Above this time there was a gradual divergence of results so that for cultures incubated for 200 hours the Celloscope gave values which were 50 times as large as those obtained by the plate dilution method One obvious reason for this discrepancy is the fact that the apparatus counts all particles both viable and dead and the plate dilution method records only viable cells

When the size distribution of *E coli* was determined in the particle counter the appearance of the distribution was found to depend on the age of the culture After 4 hours incubation the distribution could be characterized as more or less normal with a mean size of 1 03 microns After 200 hours incubation on the other hand the same culture showed two peaks on the differentiated form of the distribution curve one at 1 03 microns as before and another at 0 90 microns It is possible that the latter peak was caused by dead bacteria which had not yet disintegrated It is reasonable to assume that the conductivity of the bacteria increases before they finally disintegrate which would give rise to a smaller pulse in the instrument i e the bacteria would

be recorded as being smaller than they actually were However the distribution curve of the culture incubated for 200 hours showed that the number of cells in the populations at the two peaks with different mean sizes are approximately the same whereas the ratio of viable cells to dead cells was 1 50

The determination of the size distribution of Salmonella bacteria living or killed by KCN did not support a hypothesis that all dead cells irrespective of the circumstances will give smaller electrical pulses in the Celloscope Further studies are in progress at present with a view to investigating whether dead and viable cells really behave differently in the instrument

The necessary conditions for counting and determining the size of bacteria—i e obtaining a sufficiently low bacterial conductivity in relation to that of the suspension medium —were found to be given by 0 2–4 0 per cent NaCl for the cultures investigated At the higher concentration of NaCl i e with greater conductivity the pulse strength improved in relation to the background interference in the instrument However this differential may be attained for any concentration of NaCl by making a simple change in the electronic circuit of the amplifier All the experiments in this study were performed with physiological saline which provided satisfactory results However when making counts of bacteria which are smaller than those employed in this study it is recommended that the NaCl concentration be increased or that the above mentioned change in the instrument be made

Bacterial counting in the Celloscope is accurate within the concentration range of 30 000–600 000 cells per ml The lower limit is governed by the need to keep the suspending fluid free from extraneous particles Under exceptionally favourable conditions counts may be made at considerably lower bacterial concentrations The upper limit of bacterial concentration is governed by the uncertainty of correction for coincidence loss i e the reduction in the number of recorded cells because two or more particles may pass through the aperture channel at the same time and be recorded as one However since the cell concentration can always be reduced by dilution the limit drawn up by this study does not really restrict the range of the use of the instrument

SUMMARY

The numbers of bacteria in suspensions of growing *Escherichia coli* *Salmonella typhi murium* and *Streptococcus faecalis* were determined in an automatic particle counter the Celloscope 202 Results were compared with those obtained from viable counts by the plate dilution method These methods provided almost identical results as long as the age of the bacterial culture was less than 10 hours When the bacteria were allowed to grow for longer periods the particle counter gave increasingly higher figures than the plate dilution method

The determination of the size distribution in the particle counter of *E coli* after prolonged incubation showed *two population* peaks of different mean sizes

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CORRODING AND SPREADING COLONIES IN *MORAXELLA NONLIQUEFACIENS*

By

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Received 21.i.69

Growth with depressed, corroding colonies with a tendency to peripheral spread on the surface of blood agar plates was first described by *Henriksen* (5) in the organism named *Bacteroides corrodens* by *Eiken* (3). Recently it was reported that some strains of *Moraxella kingii* also grow in a similar manner (7).

In previous work with *Moraxella* species we have observed that some strains have a tendency to produce pitting of the medium under the colonies, which may be visible as a tiny groove around the margin of the colony, or which only becomes visible when the colony is removed. Also, after incubation for several days, the colonies frequently become irregular with more or less dentate or undulate margins (6). But we have not paid due attention to these phenomena until we came across an unusual strain of *Moraxella nonliquefaciens*, which is the basis of this report.

MATERIAL AND METHODS

The strains 4663/68, 6088/68, 6097/68 and 6121/68 were all isolated from blood agar cultures of nose swabs. The type strain of *Moraxella nonliquefaciens* 4663/52 (ATCC 19975, NCTC 10464) had been preserved lyophilized. (By coincidence the strain 4663/68 received the same registration number as the type strain, but in a different year.)

Cultural, biochemical and transformation tests were carried out by the methods used before (1, 2) with minor modifications.

DESCRIPTION OF STRAIN 4663/68

The primary culture contained numerous colonies which looked like colonies of *Moraxella kingii* or *B. corrodens*: small depressions in the agar of 0.5 to 1 mm diameter with a tiny smooth central papilla surrounded by a shallow moat with finely granular bottom. In subcultures young colonies maintained this appearance. On continued incubation the centre of the colonies became raised, hemispherical or more irregular, and the margins of the colonies spread peripherally

After 4 to 5 days the colonies were surrounded by one or more zones of thin finely granular film of growth (Figs 1 to 3) When the colonies were scraped off *depressions in the agar surface* corresponding to the growth became visible

In serial subcultures transferred every *4 to 5 days the strain* maintained these characteristics but there were considerable variations in the appearance of different cultures

A Search for Other Strains with Similar Growth

Three strains with the appearance of *Moraxella* strains (6088/68 6097/69 and 6121/68) were isolated within a couple of days They all had the usual appearance of *M nonliquefaciens* When these strains were subcultured and incubated for 4 to 5 days a few colonies showed *tongue like* or film like offshoots from the margins whereas the majority of colonies remained more regular On subculture of such offshoots as well *as of the smooth regular colonies each of the three strains could* be separated into two variants or dissociants one of which showed the peculiar corroding and spreading colonies of strain 4663/68 whereas the other produced more regular smooth colonies without pitting or spreading Only two of these three strains have been studied more closely and in both cases the two variants have remained stable over several months with transfer to new media every 4 to 5 days The corroding and spreading variants have shown no tendency to change to the smooth form Well separated colonies of this variant sometimes appear smooth when the depressions become covered by *bacterial growth* but when such smooth looking colonies are subcultured they produce the *corroding and spreading type of growth*

The smooth variants also appear to be stable but occasionally they have produced colonies which show offshoots and a tendency to pitting (Figs 4 to 6)

The four strains possessed all the morphological cultural and biochemical characters of *M nonliquefaciens* A difference between stab

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Figs 1–6

Fig 1 *M nonliquefaciens* strain 4663/68 3 days blood agar × 4 Smooth hemispherical centre surrounded by granular film of growth

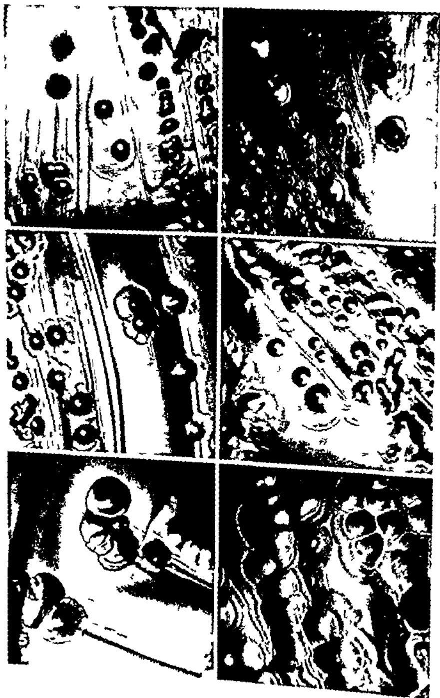
Fig 2 Same strain 4 days blood agar × 4 Raised smooth or irregular centres surrounded by irregular depressed marginal zones which have partly been filled with growth

Fig 3 Same strain 5 days blood agar × 4 Central smooth papillas surrounded by shallow moats with finely granular bottom

Fig 4 *M nonliquefaciens* strain 6088/68 5 days blood agar × 4 Colonies with raised smooth centres surrounded by granular film of growth Corroding variant

Fig 5 Same strain 5 days blood agar × 4 Smooth non corroding variant

Fig 6 *M nonliquefaciens* strain 6121/68 [illegible] days blood agar × 4 Raised centres with granular surface surrounded by concentric zones of growth with granular surface Corroding variant



cultures in semisolid swarm agar of the two variants was noted The corroding and spreading variants (including strain 4663/68) produced a woolly growth around the stab after a day or two due to a marked tendency to penetrate into the agar around the stab The smooth variants only showed this phenomenon in a reduced scale and after longer incubation

On nutrient agar plates the corroding variants produced the same type of colony as on blood agar

In order to verify that the strains really belonged to *M nonliquefaciens*, transformation tests were carried out with DNAs of streptomycin resistant single step mutants of two of the spreading variants as well as of the type strain with the streptomycin sensitive wild type of the latter as recipient The results are shown in Table 1 They indicate degrees of compatibility between the three strains corresponding to those usually found between strains of the same species There can therefore be no doubt that these strains belong to *M nonliquefaciens*

TABLE 1

Transformation of Streptomycin Sensitive Moraxella nonliquefaciens Neotype Strain 4663/62 (ATCC 1997[illegible] NCTC 10[illegible]) with DNA from Streptomycin Resistant Mutants

DNA Origin§	Quantity (μg/ml)	Streptomycin resistant colonies Number/ml	Ratio†
–	None	< 10 (0)	$<$ [illegible]8 $\times 10^{[illegible]}$
4663/68 SR	44	[illegible] $\times 10^4$	[illegible]
6121/68 SR	46	[illegible]8 $\times 10^4$	0 [illegible]8
4663/62 SR	12	3 0 $\times 10^4$	–
4663/62 SR	48	3 [illegible] $\times 10^4$	–

The procedure was essentially as previously described (1) Identical aliquots of the recipient were exposed to each DNA for [illegible] minutes in *fluid medium* DNA uptake was terminated with DNase Aliquots of the mixtures were then spread on blood agar and incubated for phenotypic expression of streptomycin resistant transformants during 6 hours Thereafter 50 μg of streptomycin per ml of agar was added by diffusion from below and incubation *continued* for another 48 hours before counting of resistant colonies Except for absence of DNA the control parallel (line 1) was analogous in all respects

§ SR = streptomycin resistant mutant selected in a single step at 500 μg of streptomycin per ml

† Number of resistant colonies divided by the number appearing in intrastrain transformation at DNA saturation (line [illegible])

DISCUSSION

The study of the strain 4663/68 indicates that the ability to produce corroding and spreading colonies is not a unique property of *M kingii* and *B corrodens*, but also can occur in *M nonliquefaciens* Further more the fact that corroding and spreading variants could be isolated from three randomly selected strains of the same species in fact the first three strains studied with this point in mind (later we have seen additional similar strains) suggests that this may be a common

ability in this species. It deserves study whether the same phenomenon can be demonstrated in other *Moraxella* species. The apparent stability of the two variants is surprising in view of the ease with which the corroding variants could be isolated from recently isolated strains.

The mechanism behind this remarkable manner of growth is unknown, but it is tempting to suggest that some kind of motility might be the explanation. This is also suggested by the appearance of stab cultures in semisolid agar. It may be recalled that *Piechaud* (10) claimed to have demonstrated the type of motility first demonstrated in *Acinetobacter* species by *Lautrop* (9) in all species of *Moraxella*. *Halvorsen* (4) on the other hand failed to detect this motility in *M. nonliquefaciens*. A renewed study of this question, perhaps including a comparison of the spreading and smooth variants, might be rewarding.

It is interesting that *M. nonliquefaciens* and *M. kingii* share the property of producing spreading and corroding variants, but before any conclusions can be drawn from this fact it will be necessary to find out how widely distributed this peculiar property is among the bacteria.

SUMMARY

A strain of *M. nonliquefaciens* growing with corroding and spreading colonies like those found in *M. kingii* is described. Variants with spreading and corroding colonies were isolated from three randomly selected strains of the same species.

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SUBDIVISION OF *M TUBERCULOSIS* BY MEANS OF BACTERIOPHAGES

With Special Reference to Epidemiological Studies

By

INGA BAESS

Received 18 I 69

Phages that lyse *M tuberculosis* were isolated for the first time in 1954 by *Froman et al* who also reported that the strains examined showed different phage patterns Differences in the phage susceptibility of *M tuberculosis* strains were demonstrated in subsequent works (*Takeya et al* 1959 *Murohashi et al* 1963) particularly in a study by *Sugita et al* (1965) who found that some strains of *M tuberculosis* were resistant to a phage that lysed the other strains examined

The value of mycobacteriophages in epidemiological studies was illustrated by *Baess* (1966) with a material of tuberculosis patients from whom *M tuberculosis* was isolated in this laboratory The phage BK_1 used in that study divided the *M tuberculosis* strains into a small group with phage susceptibility and a larger group with phage resistance It was shown that except for a few cases all strains from patients with presumed epidemiological connection were either phage susceptible or phage resistant *Tokunaga et al* (1968) found similar results in a study where strains of *M tuberculosis* from patients with epidemiological connection were lysed in the same way in contrast to strains from patients with varying sources of infection where the phage susceptibility was different

The aim of the present study is to give the results obtained with a larger material comprising 230 strains of tubercle bacilli from 93 groups of patients with a presumed epidemiological connection within each group

Subdivision of the two groups of *M tuberculosis* would be of great value in epidemiological studies and therefore a large number of phages were tested on *M tuberculosis* The majority of these phages were isolated in this laboratory and some were received from other workers A few of the latter were adapted to other host strains The results with

The writer is grateful to the chiefs of the various tuberculosis dispensaries for information regarding the epidemiological connections Special thanks are due to Dr *Birte Groth* County of Copenhagen Tuberculosis Dispensary Lyngby for her great interest and assistance

seven phages are given here Since the experiments were performed with different phage concentrations they provide information concerning the importance of the choice of test concentration for the outcome of phage typing This aspect is mentioned further in the discussion

MATERIAL AND METHODS

Bacterial strains The strains from patients with epidemiological connection were chosen on the basis of information provided by the Danish tuberculosis dispensaries and the central register of Danish tuberculosis patients kept at the Statens Serum institut The writer has gone through the case histories of all the patients concerned The strains from humans were isolated at this laboratory and those from animals at the State Veterinary Serum Laboratory The material which originates from all parts of Denmark including Greenland consists of 205 strains of *M tuberculosis* and 25 strains of *M bovis* from 203 humans and 22 animals (one strain per individual) It comprises 93 groups of from two to nine strains with presumed epidemiological connection The bacteria were maintained on Lowenstein Jensen medium

Bacteriophages BK_1 isolated at this laboratory has been described previously (*Baess* 1966)

D29a was adapted to *M tuberculosis* $\times$12480 from D32 which was received from Froman (*Froman* 1954) D32 did not lyse *M tuberculosis* in the test concentrations usually employed but produced a few plaques in concentration 10^8 particles per ml on $\times$12480 After five successive transfers from one of these plaques on $\times$12480 the phage (designated D29a) was concentrated using $\times$12480 as host strain That strain was lysed by BK_1

D98a was produced in the same way from D28 (*Froman* 1954) using *M tuberculosis* E11171 as host strain That strain was not lysed by BK_1

D34 and D56 were received from Froman (*Froman* 1954 *Bogen & Froman* 1959)

D50A and GS4F were received from Redmond (*Redmond* 1963)

Redmond s phage BG_1 was received from Bates (*Bates* 1967)

Media The phages were suspended in *heart infusion* broth to which was added 1 per cent tryptose 0 5 per cent sodium chloride and 0 001 M calcium chloride The experiments were performed by the agar layer method Two different media were used *in* BC and RV 429a The BG *medium is* Bordet Gengou agar base with proteose peptone and a semi fluid medium with yeast extract and tryptone (*Froman* 1954 *Froman & Scammon* 1964) RV 429a was described by Redmond (Redmond & *Ward* 1966) and consists of RVA17 with the changes mentioned in the postscript to his article The semi fluid medium used in this study was RV 429a but with *only* 0 8 per cent agar added

The reason for using two different media was that RV 429a which had not been described at the time the experiments were commenced solved the problems in solved in phage typing of the *M bovis* strains and 23 of the *M tuberculosis* strains which grew badly on the BC medium.

Methods 1 ml of a Dubos culture of *M tuberculosis* or 1 5 ml of *M bovis* in a concentration of about 1 mg semi dried weight per millilitre was mixed with *semi* fluid medium taken from waterbath at 5° The mixture was distributed evenly over the medium in the petri dishes After solidifying up to eight different phage dilutions were applied to the plates by means of platinum needles suspended loosely from a metal plate The platinum loops used provided about 70 drops per millilitre of phage suspension When the drops had dried the *plates* were incubated at 37 and after two days put into plastic bags to avoid desiccation

The RV 429a plates were inspected for the first time after five days and the BG plates after eight days *Double* determinations were made systematically The results were recorded as confluent lysis semi confluent lysis single plaques no lysis Any kind of lysis is called + in the tables Where the results of double determination of the same strain were divergent they are recorded as ± Such results occurred only with the lowest phage concentrations by which bacteria were lysed.

Table 1 shows the number of bacterial strains included in the experiments together with the various phages the test concentrations and the media used The 104 and 30 strains were part of the total material of 230 *strains* The 104 strains

TABLE 1

Phages Phage Concentrations Number of Bacterial Strains and Media Used

No of strains	Phages	Phage concentration	Medium
230	Bk_1	10^6	BG RVA29a
230	Bk_1	$10^9 \rightarrow 10$	BG RVA29a
230	GS4F	$10^7 \rightarrow 10^1$	RVA29a
104	D56	$10^8 \rightarrow 10$	BC
104	D34	$10^7 \rightarrow 10^1$	BC
30	DS6A	$10^7 \rightarrow 10^0$	RVA29a
30	D28a	$10^6 \rightarrow 10^3$	BG
30	D32a	$10^7 \rightarrow 10^3$	BC
30	BG_1	$10^8 \rightarrow 10^1$	RVA29a

Part of material published in *Baess* Amer Rev *resp Dis* 93 1965

were those available at the time phages D56 and D34 were examined and the 30 were selected because of their good growth and because they represented a reasonable distribution of strains lysed by Bk_1 and strains resistant to Bk_1 Bk_1 and GS4E were tested on all 230 strains and all phages were tested on the 30 strains

RESULTS

Epidemiological examination According to the case histories and information from the tuberculosis dispensaries the 230 strains examined could be distributed on the basis of presumed epidemiological connection into groups as shown in Tables 2 and 3 There were 88 groups of *M tuberculosis* one originating from one human and four animals Four of the five groups of *M bovis* were from both humans and animals

TABLE 2

Distribution of 205 Strains of M tuberculosis According to Presumed Epidemiological Connection

Epidemiological connection	No of groups	No of individuals
Married couples	11	22
Parents and children	34	71
Brothers and sisters	4	8
Grandparents and grandchildren	8	16
Other relations	6	13
Adults and children not related to each other	5	11
Persons with same residence	2	4
Persons with same place of employment	[illegible]	4
Neighbours or acquaintances	[illegible]	4
Mixtures of above connections	8	3[illegible]
Medical staff and patients	2	4
Humans and animals	1	5
Persons with contact according to data from tuberculosis dispensaries	3	7
Total	88	205

TABLE 3

Distribution of 25 Strains of M bovis According to Presumed Epidemiological Connection

Epidemiological connection	No of groups	No of individuals
Humans and animals	4	23
Brothers in law with same residence	1	2
Total	5	25

The assumption of epidemiological connection was supported in nine groups by the finding of resistance to streptomycin PAS or INH or combinations of those drugs all the strains in the group having the same drug resistance pattern

In eight other groups the epidemiological connection was confirmed by the finding of poor growth on BG medium so that only RVA29a could be used for testing

In two groups each of two patients the epidemiological connection was refuted by the different morphology of the bacterial colonies in the one case and the different growth on BG medium in the other This finding was confirmed by the results of phage typing (see below)

TABLE 4

Phage Typing of M tuberculosis by Different Phage Concentrations

Phage		Concentration 10^8	10^7	10^6	10^5	10^4	10^3	10	101
Bh_1	+			62					
	±			0					
	—			143					
BK_1	+	87	67	64	62	57	43	9	
	±	13	8	3	0	5	7	14	
	—	10?	130	138	143	143	155	182	
C54C	+		133	75	62	62	49	9	0
	±		26	30	4	0	11	21	1
	—		46	100	139	143	145	175	204
D56	+	90	8	4	2	1	0	0	
	±	3	1	0	0	1	0	0	
	—	81	95	100	109	109	0	0	
D34	+		5	2	2	1	1	0	0
	±		1	0	0	1	0	0	0
	—		98	109	109	109	103	104	104

± Divergent results in double determinations (occurred only in the lowest phage concentrations by which the bacteria were lysed)
Same strains

In the other groups the results of the prior bacteriological examinations were so uniform that they neither confirmed nor refuted the clinical evaluation of the epidemiological connection

The two experimental series with Bk_1 divided the 205 *M tuberculosis* strains into 62 phage susceptible (30 2 per cent) and 143 phage resistant (69 8 per cent) (Table 4) When distributed into the 88 patient groups 22 (25 per cent) contained only phage susceptible and 61 (69 3 per cent) only phage-resistant strains Five (5 7 per cent) contained both phage susceptible and phage resistant strains Thus in five groups there is discrepancy between the clinical evaluation of the epidemiological connection and the result of phage typing

One of these five groups consisted of four phage susceptible strains and one phage resistant A renewed request to the tuberculosis dispensary revealed that it is most likely that the patient with the phage resistant strain was discovered accidentally during examination of environments

Another of the groups consists of two adult brothers from Greenland On account of the wide extent of the disease in that part of the country it is possible that each can have had his own source of infection Furthermore the strains did not resemble each other morphologically

A third group consists of a mother and son The mother has pulmonary processes of older date The son is a fisherman and an alcoholic with a large circle of acquaintances One of his associates had tuberculosis at the same time but it has not been possible to test the strain from that patient

Two patients in the fourth of these groups are from families in close contact with each other However the strains did not resemble each other as regards growth since only the one grew well on BG medium

The last of the five groups is a married couple and in this case the discrepancy between the epidemiological evaluation and phage typing cannot be explained

Further evaluation revealed that the phage susceptibility of *M tuberculosis* to Bk_1 seems to be independent of the age sex residence and BCG vaccination of the patient and the stage localization or course of the disease Neither does it have any relation to the colony morphology of the bacteria the drug resistance (also to furfuroyl 2 hydrazine) or the result of the niacin test

Phage typing of the *M bovis* strains is not possible since all were resistant to Bk_1 (not shown in tables)

Phage CS4E subdivided *M tuberculosis* in the same way as Bk_1 the same 62 strains being lysed by the phage in concentration 10^4 (Table 4) As with Bk_1 the *M bovis* strains cannot be subdivided since none of them are lysed by GS4L in concentrations below 10^6 (not shown in tables)

D56 lysed two out of 104 strains in concentration 10^5 and four in concentration 10^8 The lysis of one of the latter strains took the form

TABLE 5

Phage Typing of M tuberculosis by Different Phage Concentrations

Phage		Concentration 10^8	10^7	10^6	10^5	10^4	10^3	10	10^1	10^0
Bh_1	+	6	6	6	6	4	3	0		
	±	2	0	0	0	2	0	1		
	—	22	24	24	24	24	27	29		
GS4E	+		12	6	6	6	5	1	0	
	±		5	5	0	0	1	2	0	
	—		13	19	24	24	24	27	30	
BC_1	+	30	28	27	13	6	2	0	0	
	±	0	1	0	4	0	4	0	0	
	—	0	1	3	13	24	24	30	30	
D32a	+		13	6	6	6	5			
	±		7	0	0	0	1			
	—		10	24	24	24	24			
DS6A	+		30	30	30	30	24	4	*0*	*0*
	±		0	0	0	0	6	*8*	*1*	*0*
	—		0	0	0	0	0	18	29	30
D28a	+			30	30	30	29			
	±			0	0	0	1			
	—			0	0	0	0			
D36	+	5	3	2	0	0	0	*0*		
	±	2	0	0	0	0	0	*0*		
	—	23	27	28	30	30	30	30		
D34	+		3	2	2	1	1	0	0	
	±		0	0	0	1	0	0	0	
	—		27	28	28	28	29	*30*	*30*	

± *Divergent results in double determinations (occurred only in the lowest phage concentrations by which the bacteria were lysed)*
Same strains

of turbid partially confluent plaques The four *strains* form two groups each containing two patients with *epidemiological* connection One of the groups was lysed by D36 only and the other also by Bh_1

D34 contributes nothing to the subdivision of *M tuberculosis* since in concentrations 10 and 10^4 the *phage* lysed only two out of *104* strains producing small *turbid* plaques These two strains *belong* in an *epidemiological group* of four patients

BC_1 and the adapted phage *D32a* subdivide in the same way as Bh_1 and *GS4E* since both lysed the same six of the 30 strains examined (Table 5)

DS6A and the adapted phage *D28a* lysed all the 30 *M tuberculosis* and thus are of no significance in an epidemiological examination of this kind

Test concentrations of the phages It will be seen from Tables 4 and 5 how different are the results obtained by phage typing using varying concentrations Table 5 shows that the four phages which divide 30 strains of *M tuberculosis* into 6 phage susceptible and 24 phage resistant do so within varying concentration ranges viz Bh_1 and D29a in three dilution stages GS4E in two and BG in only one The number of phage susceptible strains becomes greater as the concentration increases and the number of phage resistant strains becomes greater as the concentration decreases This can be seen most clearly as regards Bh_1 and GS4E from the larger material shown in Table 4 Here the presumed correct distribution occurs at only one concentration The increase in the number of phage susceptible strains occurs most rapidly with phage GS4E Even though the majority of the experiments with Bh_1 were performed on BG medium and those with GS4E on BV29a medium the results are comparable Control experiments have shown that the host ranges of the two phages are the same and that they lyse the bacteria to the same degree on both media As is the case with the other phages DS6A which lyses all the *M tuberculosis* strains in concentrations above 10^3 lyses fewer strains as the concentration decreases (Table 5)

Subdivision of the strains with *D34* and *D56* is only seemingly similar since it is not the same strains that are lysed by both phages

Bh_1 did not lyse any *M bovis* strains in concentrations up to 10^8 GS4E lysed one strain of *M bovis* on one of the two similar plates in concentration 10^8 and five strains in concentration 10^7 (not shown in the tables)

DISCUSSION

The most frequent finding in experiments with phage typing of tubercle bacilli is that all the strains examined show either complete resistance or complete susceptibility to the individual phages employed *Froman et al* (1954) described phage patterns for *M tuberculosis*, but their findings could not be reproduced by the writer

A few works mention differences in the phage susceptibility of *M tuberculosis* strains but such findings have not been used in solving epidemiological problems *Takeya et al* (1959) found the phage susceptibility of virulent strains to be greater than that of less virulent strains and they assumed the reason to be the varying colony morphology *Murohashi et al* (1963) demonstrated a difference in the degree of lysis with phage GS4E in a concentration of 5×10^6

As mentioned previously the use of mycobacteriophages in epidemiological studies of patients with tuberculosis was described for the first time by *Baess* in 1966 In the present work the same phage (Bh_1) was used to examine a larger material consisting of 205 strains of *M tuberculosis* from 88 groups of patients with a presumed epidemiological connection within each group It was shown by this means that Bh_1

vided the 205 strains into 62 phage susceptible and 143 phage resistant When distributed into the 88 patients groups it was seen that 22 were infected with phage susceptible 61 with phage resistant and 5 with both phage susceptible and phage resistant strains *Thus a connection must exist between epidemiology and phage susceptibility* since with a pair wise fortuitous distribution of the strains according to the frequency of phage susceptibility found in the present work about 40 per cent of the groups (i e 35) would be infected with both phage susceptible and phage resistant strains

As stated earlier *Tokunaga et al* (1968) obtained similar results in a material comprising two patient groups Using geographical distribution of the strains *Bates* (1967) was not able to contribute to a solution of epidemiological problems This would not have been possible in the present work either had geographical classification alone been used

It would seem from the present study that the phage type in *M tuberculosis* is a constant characteristic The experiments with Bk_1 and GS4E have stretched over a period of about three years during which the strains have been subcultured six times Despite this the results have been absolutely uniform In a few cases the examination of more than one strain from the same patient—some isolated from different organs —showed systematically either susceptibility or resistance to Bk_1

This constancy of the phage type is of special importance in this study for evaluation of the five patient groups from which both phage susceptible and phage resistant bacteria were isolated Based on the assumption of this constant characteristic these five groups must have been erroneously evaluated and therefore fortuitously composed as regards phage susceptibility As mentioned above about 40 per cent of such strains randomized pair wise would show different phage susceptibility and thus be recognized as being erroneously grouped Therefore the five groups represent only about 40 per cent of all the groups evaluated incorrectly The remainder of the errors must be in the other groups the majority being among the phage resistant strains of which there are most

It should be stressed that clinical evaluation of the epidemiological connection between two tuberculosis patients may be complicated by the long interval between the infection and manifestation of the disease Such a situation may explain the discrepancy between the ability of the strains to become lysed in two adult patients who become ill simultaneously in circumstances that make epidemiological connection possible Erroneous evaluation has also been found previously in this department by means of type determination of tubercle bacilli where the one patient was shown to be infected with *M tuberculosis* and the other with *M bovis* (*Engbæk* 1969)

However it cannot be excluded completely that a change in phage type may occur so that the clinical epidemiological evaluation is correct in spite of the result of the phage examination It is known that for

instance in staphylococcus epidemics the phage type may become changed by lysogenization of the bacteria.

Efforts to subdivide *M. tuberculosis* by means of other phages showed that GS4E, BG_1 and D32a divided the strains into the same two groups as Bk_1. GS4E was tested on all the material (Table 4) and the other two phages on only 30 strains (Table 5). It is also known from the study of *Bates* (1967) that GS4E and BG_1 lyse the same strains of *M. tuberculosis* and *Tokunaga et al.* (1968) found that Bk_1 and GS4F resemble each other. It is noteworthy that three of these phages with the same host range have such widely differing host strains as *M. tuberculosis*, Battey bacillus and *M. smegmatis*.

DS6A and D28a lysed all strains of *M. tuberculosis* and therefore are of no assistance in subdivision (Table 5).

D32a, which lyses some, and D28a, which lyses all strains of *M. tuberculosis*, are adapted to a Bk_1 susceptible and a Bk_1 resistant strain respectively. However, GS4E and DS6A, which show the same difference in lysis as D32a and D28a respectively, have the same Bk_1 susceptible *M. tuberculosis* strain as host. This is not in accordance with the assumption that the difference between D32a and D28a is due to a host induced modification.

Since D56 in concentration 10^6 lysed the strains in two groups of patients with epidemiological connection, this phage may contribute to a subdivision of *M. tuberculosis*. D34 lysed only few strains of *M. tuberculosis*, and since the lysis was turbid and weak, its value in epidemiological studies is only limited. *Bates* (1967) is more optimistic as regards D34 but does not mention the actual phage concentration used or the character of the lysis. It is possible that small differences in medium may explain the divergent results with D34 in Bates's and the present work.

The results in the present study indicate that it is very difficult to subdivide *M. tuberculosis* into other than the two groups achieved with Bk_1. D56 can be used tentatively in supplementary experiments in connection with epidemiological studies. This agrees in principle with the findings of *Tokunaga et al.* (1968).

It can be seen from Tables 4 and 5 how important the choice of phage concentration is for the result of the experiments, since a shift of only one stage in a tenfold dilution series can give different results.

It has long been accepted that high phage concentrations are not suitable for phage typing. One could perhaps agree *to select a constant*ly lower phage concentration for all phages and accept the subdivision of the bacteria thus obtained, as has been done by *e.g. Baess* (1969). However, it will be seen from Tables 4 and 5 *that this is not an ideal* solution. It is still an unclarified question as to whether the RTD can be used for mycobacteria since the size of the plaques varies considerably dependent on the phages used. *The RTD for phages* with the smallest plaques lies around 10, which is near the concentration at

which lysis from without can be seen Furthermore the rate of growth of the host strains varies often quite considerably from that of the bacteria tested which influences the size of the plaques and which in turn affects the RTD Should the RTD be determined on a strain of the species to be tested or on the host strain of the phage? In any event if the RTD is used for mycobacteria very varying test concentrations would be used for testing bacteria whose growth rate often deviates considerably from that of the host strain on which the test concentrations are based

A time consuming and difficult resort is to check the various dilutions on a series of mycobacteria with the same growth rate and possibly of the same species as those to be tested (Table 5) The results would then indicate in which concentration range the phage can be used and how wide that range is

It will be seen from Table 5 that the first four phages which subdivide the bacteria in the same way are not equally appropriate for the purpose BK_1 and D32a are the most suitable since they show concordant results in the widest concentration range Table 4 shows that the suitable concentration rate of BK_1 is narrowed down in the larger material It can also be seen that in the first experiment the strains could be divided into 62 phage susceptible and 143 phage resistant by concentration 10^6 but only by concentration 10 in the second attempt This would indicate a reduction in the suitability of BK_1 Since phages from a possibly lysogenic host strain should not still be able to manifest themselves by diluting to 10^6 and 10 this is probably on account of admixture with mutants with a different host range This could also be the explanation for the poorer quality of GSH and BG_1 In that event the phages could be improved by propagation from single plaques It is not likely that the variations in the results of phage typing by the different concentrations are due to the inhomogeneity of the bacterial strains If that were so it could be expected that it would be the same phage concentrations that would give similar subdivision of the bacteria

If in testing a strain a doubtful result is obtained with one phage concentration because of limitation of the suitable concentration range tenfold dilutions of the phage can be used for comparing two bacterial strains In this way it can be seen whether the two strains are lysed in the same way or differently since there will be the same trend in the decrease of lysis of similar strains

SUMMARY

The present material is an extension of that reported in a previous study from 1966 It now comprises 205 strains of *M tuberculosis* and 25 strains of *M bovis* consisting of 88 and 5 groups of patients with presumed epidemiological connection from the two species respectively Phage BK divided the 205 strains of *M tuberculosis* into 62 phage susceptible and 143 phage resistant When distributed into the 88 patient

groups 22 contained only phage susceptible and 61 only phage-resistant strains. Five groups contained both phage susceptible and phage resistant strains. Thus a connection must exist between epidemiology and phage susceptibility. All the *M. bovis* strains were phage resistant.

In addition to BK₁ the lysis of *M. tuberculosis* by a number of other phages was examined in an attempt to further divide the two subspecies. The results with seven of the phages indicate that it is very difficult to subdivide *M. tuberculosis* into other than the two groups achieved with BK₁. D56 can be used tentatively in supplementary experiments in connection with epidemiological studies.

Comparative examination of all the eight bacteriophages in tenfold dilutions shows that it is necessary to be critical in selecting the phage concentrations for use in testing mycobacteria. It is possible that instead of the RTD the phage concentration used should be that determined by tenfold dilutions on mycobacteria of the same species or having the same growth rate as those to be tested. Furthermore the experiments indicate that it is necessary to check the purity of the phages from the point of view of admixture with mutants or phages from lysogenic host strains.

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ANTIGENIC DETERMINANTS OF AQUEOUS ETHER EXTRACTED ENDOTOXIN FROM *NEISSERIA GONORRHOEAE*

By

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Previous investigations showed that the crude preparations obtained by extraction of gonococci with aqueous ether alkali trichloroacetic acid or heating contained an antigenic polysaccharide determinant called *a* and a protein determinant called *b* (11) Purified phenol water lipo polysaccharide possessed the activity of *a* but not that of *b* These findings were based on the indirect haemagglutination and haemag glutination inhibition techniques It was suggested that the *a* deter minant was associated with the polysaccharide component of the endotoxin of gonococci whereas determinant *b* may be associated with the protein component

The aim of the investigations described in this paper was two fold 1 A study of the relationship between the determinants *a* and *b* 2 Isolation and purification of these determinants The aqueous ether extract of gonococci was selected for these studies

MATERIALS AND METHODS

Neisseria gonorrhoeae strain 8551/64 was employed The methods for cultivation and harvesting of the bacteria and for preparation of antiserum to whole gonococci (anti Gc) have been described earlier (9)

Extraction with aqueous ether was carried out as earlier (11)

Serological Methods

Sensitization of sheep erythrocytes Preparations which sensitized erythrocytes with determinant *a* were prepared as follows Aqueous ether endotoxin was treated with 0 04 N NaOH at 37 C for 18 hrs followed by neutralization with HCl and dia lysis against 1/15 M phosphate buffered saline pH 7 2 The preparation was then digested with pronase The enzyme was inactivated at 100 C for 5 mins

Preparations which sensitized erythrocytes with determinant *b* were prepared by treatment of the endotoxin with 0 008 N NaOH at 37 C for 18 hrs The neutralized preparation was oxidized with periodate to destroy determinant *a* dialysed against running tap water and finally against buffered saline

For sensitization equal volumes of a one per cent suspension of sheep erythro cytes and an antigen solution were mixed The solution contained 4 times the least

amount of antigen sensitizing *erythrocytes to* maximal agglutination titres with antisera

The experiments which led to these procedures will be described in another paper (12)

The indirect haemagglutination test and test for inhibition of haemagglutination were performed according to earlier *reports (9 11)* The serological activity of *a and b* was *determined by the inhibition test* Minimal inhibiting dose (MID) was the least amount of a preparation (in μg) which completely inhibited 8 agglutinating *units of the antiserum (8 times the* amount of antiserum corresponding to the titre)

Preparation of specifically absorbed antisera Antiserum containing antibodies to *a* (anti Gc *a*) *but not to b* was prepared by absorption of anti Gc with gonococci treated with *periodate The treatment* was *carried out by* suspending one gram of wet bacteria in 5 ml of 0 04 M sodium metaperiodate The suspension was kept in the dark at 20 C for 20 hrs The packed and washed *bacteria were then used for* absorption of 0 5 ml of anti Gc Antiserum containing antibodies to *b* (anti Gc *b*) but not to *a* was prepared by absorption of anti Gc with purified phenol water endotoxin (11)

Complete absorption was checked by testing a 1 2 dilution of the absorbed sera against appropriately sensitized erythrocytes

Isolation of endotoxin by specific precipitation was carried out as follows A solution of the endotoxin was centrifuged at 1500 × *g* for 15 mins MID of the supernatant was determined and equivalent volume were added to 0 2 ml of un diluted anti *Gc a* and anti *Gc b* After incubation at 4 C for 48 hrs the precipitates were collected by centrifugation at 1000 × *g* for 15 mins and washed 3 times The washed precipitates were suspended in a small volume of buffered saline *heated in* a boiling water bath for 2 mins to denature the antibodies and were then tested *for serological activity*

Precipitation by ring test and by gel diffusion were performed according to the techniques described previously (10)

Fractionation Methods

Ultracentrifugation was carried out in the Spinco Model L 50 preparative ultra centrifuge

Treatment of the endotoxin with aqueous phenol was performed by mixing equal volumes of an aqueous solution containing 2 mg of endotoxin per ml *and 90 per* cent phenol The mixture was stirred at 37 C for 5 mins cooled to 4 C and centrifuged The water phase was withdrawn dialysed against *running tap* water and lyophilized Four volumes of absolute ethanol were added to the phenol phase The mixture was *kept at 4 C for 6 to* 24 hrs and was *then centrifuged* The sediment was taken up in water dialysed against running tap water and lyophilized

Paper electrophoresis was *performed* at room temperature using veronal buffer pH 8 6 ionic strength 0 1 or a phosphate buffer pH 12 ionic strength 0 1 Ten to 20 *μl of a one per cent* solution of the endotoxin were applied to the strips A current of 8 mA and 60 V the voltage gradient being 2 6 V cm⁻¹ was applied for 6 to 16 hrs The paper strips were stained for protein with bromphenol blue

Block electrophoresis was performed using Pevikon C 870 (Stockholm *Superfosfat* Fabriks AB Stockholm Sweden) according to the method of *Fahey & McLaughlin* (4) The pevikon block was prepared in a plexi glass tray *measuring 16 × 27 × 0 5* cm A phosphate buffer pH 12 ionic strength 0 06 was used The electrophoresis was carried out for 14 hrs at 4 C and a const*ant voltage of 300* V and 50 mA was applied After the electrophoresis the block was divided into 1 5 cm wide sections Each section was suspended *in 5 ml* of phosphate buffered saline pH *7 2 and cen*trifuged at 1000 × *g* for 15 mins The supernatants were dialysed against buffered *saline for 48 hrs and the* optical density serological activity and DNA *content were* determined

Gel filtration of 10 to 20 mg of endotoxin was carried out on columns of Sephadex G-200 of 2 5 × 50 cm according to *Flodin* (5) The columns were *prepared and* eluted either with 0 05 M phosphate buffer pH 7 4 or with *0 06 M phosphate* buffer pH 12 with a flow rate of 20 ml per hour The eluate was *collected in* 10 ml fractions and the optical density at 280 mμ was determined After dialysis against water the serological activity and DNA content of each fraction were determined

Chemical Analyses

Neutral sugar was estimated by the sulphuric acid orcinol method (20). Glucose galactose 1:1 was used as standard.

Protein was determined by the Folin Ciocalteu method according to *Lowry et al.* (8) with bovine serum albumin as standard.

Lipid was determined by the method of *Snyder & Stephens* (17) as modified by *Tauber* (18) with tripalmitin as standard.

Nucleic acids were examined spectrophotometrically at 260 mμ. DNA was estimated by the diphenylamine reaction of *Dische* (3). DNA from calf thymus (Sigma Chemical Company) served as standard.

Other Methods

Oxidation with periodate and *digestion with pronase* were carried out as described earlier (11).

Treatment with DNase (DNase 1, B grade, Calbiochem) of the crude *endotoxin* was carried out in 0.1 M acetate buffer pH 5.5 at 37° C for 60 mins. *The enzyme to* substrate (DNA) ratio was 1:50 (W/W).

The epinephrine skin test was employed to demonstrate endotoxic activity according to the procedure described previously (11).

EXPERIMENTS AND RESULTS

Samples containing 2 to 10 mg per ml of crude endotoxin in water were centrifuged at 60 000 × *g* for one hour. The *supernatants* contained great amounts of nucleic acids whereas the *determinants a* and *b* were quantitatively sedimented. The *pellet* was washed once with water and the final sediment was *lyophilized*.

In the gel diffusion tests both the crude extract and the supernatant after centrifugation formed a *precipitation line* with anti Gc midway between the antigen and the serum *basins*. The antigen giving rise to this precipitation line was destroyed by heating at 100° C for 15 mins. Since determinants *a* and *b* are thermostable (11) and were sedimented by ultracentrifugation, this precipitinogen must be different from *a* and *b*. No other *precipitation* lines were observed when concentrations of up to 10 *mg per ml* of the crude preparation were applied. The use of 0.5 per cent of agar instead of one per cent, incubation at different temperatures and filling of the antigen basin for up to 20 hrs before application of the *antiserum* did not result in the appearance of other precipitation lines. The ultracentrifuged endotoxin formed no precipitation line with the *antisera*. However, this preparation reacted with anti Gc in the *ring test*. The preparations obtained after destruction of *a* by periodate *oxidation* or of *b* by pronase digestion also gave a positive ring test.

Ultracentrifuged *and* washed preparations contained from 5 to 8 per cent of DNA. In an attempt to separate and purify the protein and the polysaccharide components, the preparation was subjected to electrophoresis, *gel filtration*, treatment with aqueous phenol and precipitation with specific *antisera*.

On paper electrophoresis in *veronal* buffer (pH 8.6) no migration of the material occurred. Using the phosphate buffer (pH 12), the paper

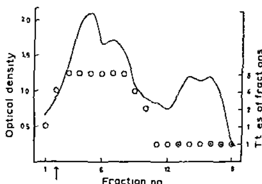


Fig 1

Fractionation by Pevikon block electrophoresis of 25 mg of ultracentrifuged endotoxin Phosphate buffer pH 1? ionic strength 0 06 and electrophoresis for 14 hrs.
—— Optical density at 280 mμ
Titres in the haemagglutination inhibition test of determinant *a*
○ Titres in the haemagglutination inhibition test of determinant *b*
Arrow indicates application of the sample Anode to the right

was stained by bromphenol blue over an area covering various distances from the origin towards the anode depending on the duration of the electrophoresis In order to study more thoroughly the behaviour of the endotoxin in electrophoresis 25 mg of endotoxin was subjected to block electrophoresis in the phosphate buffer The optical density at 280 mμ and the serological activity of the 18 fractions obtained are demonstrated in Fig 1 The UV absorption curve shows two maxima corresponding to fractions 5 and 16 Materials inhibiting haemagglutination were found in fractions 1 to 10 showing that the bulk of the endotoxin had migrated towards the anode The distribution over this wide area agrees with the results obtained by paper electrophoresis and indicates heterogeneity of the endotoxin A rough parallelism existed between the optical density of fractions 1 to 10 and the serological activity of these fractions The proportionate activity of *a* and *b* was the same in nearly all the fractions and the same as that of the corresponding crude preparation This shows that no separation of the components had been achieved Nucleic acids were only found in fractions 12 to 18 and were thus separated from the endotoxin Fractions 1 to 10 were combined and lyophilized However the preparation contained substantial amounts of pevikon particles Probably due to this contamination the serological activity of the dried material was somewhat lower than that of the corresponding crude preparation

After treatment of the ultracentrifuged endotoxin with aqueous phenol the water phase contained nucleic acids but did not react with antisera in the serological tests The material recovered from the phenol phase contained both the determinants *a* and *b* but no nucleic acid showing that purification had been obtained Using erythrocytes sensitized with the *a* determinant the minimal inhibiting dose of this

preparation was 6 25 μg i e half the amount needed of the crude preparation (12 5 μg) The activity of a was not influenced by exposure of the preparation to phenol for up to 24 hrs However the activity of determinant b was lower than that of the crude preparation Prolonged exposure of the preparation to phenol resulted in a proportional decrease of the activity of b The preparation which had been left in contact with phenol for 24 hrs had no longer the activity of determinant b The loss of the activity of b was probably due to a denaturing effect of the phenol or phenol alcohol mixture

In gel filtration of the ultracentrifuged endotoxin essentially similar results were obtained with the two buffers pH 7 4 and pH 12 The preparation was soluble at pH 12 but formed an opalescent suspension at pH 7 4 Both the determinants a and b and the DNA were eluted in the fractions corresponding to the void volume of the column Only the serologically active fractions absorbed UV light at 280 mμ Neither separation of a and b nor further purification of the preparation was obtained by gel filtration

Attempts were made to isolate the endotoxin by precipitation with anti Gc a and anti Gc b A precipitate was obtained with both antisera whereas incubation of the antigen with normal rabbit serum gave no precipitation The precipitates were collected and washed by centrifugation at low speed (1000 $\times$ g) in order to avoid sedimentation of free antigen Some of the precipitates were lost probably due to this low speed After the antibodies bound in the precipitates had been denatured by heating at 100 C for 2 mins solutions of the precipitates inhibited the agglutination of erythrocytes sensitized with the a or b determinant They also reacted on ring test precipitation both with anti Gc a and anti Gc b Each antiserum thus precipitated both a and b

Effect of Treatment of the Endotoxin with DNase

In order to remove DNA without exposure of the antigen to alkali or phenol the crude endotoxin was treated with DNase During incubation with the enzyme the antigen sedimented as a white precipitate probably because of the acidity of the buffer (pH 5 5) The precipitated antigen was collected by centrifugation at 1000 $\times$ g for 15 mins washed once with the buffer suspended in water and dialysed Compared with crude endotoxin the enzyme treated preparation showed increased tendency to aggregation and was completely sedimented by centrifugation at 30 000 $\times$ g for one hour After 3 washings with distilled water the final sediment was lyophilized with the yield of about 0 3 per cent based on wet bacteria In gel diffusion tests paper electrophoresis and gel filtration this preparation behaved exactly like the ultracentrifuged endotoxin described above

Some serological and chemical data of a crude and a DNase treated preparation are shown in Table 1 The serological activity of a of the

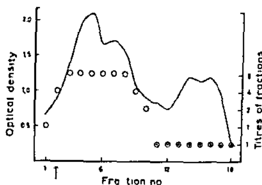


Fig 1

Fractionation by Pevikon block electrophoresis of 25 mg of ultracentrifuged endotoxin Phosphate buffer pH 7 ionic strength 0 05 and electrophoresis for 14 hrs.
—— Optical density at 280 mμ
Titres in the haemagglutination inhibition test of determinant *a*
○ Titres in the haemagglutination inhibition test of determinant *b*
Arrow indicates application of the sample Anode to the right

was stained by bromphenol blue over an area covering various distances from the origin towards the anode depending on the duration of the electrophoresis In order to study more thoroughly the behaviour of the endotoxin in electrophoresis 25 mg of endotoxin was subjected to block electrophoresis in the phosphate buffer The optical density at 280 mμ and the serological activity of the 18 fractions obtained are demonstrated in Fig 1 The UV absorption curve shows two maxima corresponding to fractions 5 and 16 Materials inhibiting haemagglutination were found in fractions 1 to 10 showing that the bulk of the endotoxin had migrated towards the anode The distribution over this wide area agrees with the results obtained by paper electrophoresis and indicates heterogeneity of the endotoxin A rough parallelism existed between the optical density of fractions 1 to 10 and the serological activity of these fractions The proportionate activity of *a* and *b* was the same in nearly all the fractions and the same as that of the corresponding crude preparation This shows that no separation of the components had been achieved Nucleic acids were only found in fractions 12 to 18 and were thus separated from the endotoxin Fractions 1 to 10 were combined and lyophilized However the preparation contained substantial amounts of pevikon particles Probably due to this contamination the serological activity of the dried material was somewhat lower than that of the corresponding crude preparation

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an area of about 13 cm shows that the preparation is composed of components with different physicochemical properties This is in accordance with the findings by *Nowolny et al* (14) By ion exchange chromatography they demonstrated heterogeneity with respect to chemical composition biological and serological properties of endotoxins from *Serratia marcescens* and *E coli*

The following findings are relevant in the evaluation of the relationship between determinants *a* and *b* 1 Both determinants were sedimented by centrifugation at different speeds 2 After treatment with aqueous phenol *a* and *b* were recovered from the phenol phase despite the fact that *a* is of polysaccharide nature 3 Separation was not obtained by block electrophoresis the proportionate activity of *a* and *b* being the same in each serologically active fraction (Fig 1) 4 Identical proportionate amounts of *a* and *b* were found in the antigen containing fractions after gel filtration 5 The precipitates formed with antisera against each component contained both *a* and *b*

These findings strongly indicate that the determinants *a* and *b* are closely associated It is therefore suggested that *a* and *b* are the respective determinants of the polysaccharide and the protein components of the aqueous ether endotoxin The nature of the linkage between these components is not known The use of pH 12 buffer in block electrophoresis and gel filtration without separation of *a* and *b* indicates stability of the linkage against alkali

Since the preparations obtained by extraction of gonococci with alkali TCA or by heating also contained *a* and *b* (11) a similar association of the components may exist in these extracts Antigenic determinants of enterobacterial endotoxins are usually considered to be of polysaccharide nature However *Homma et al* (6 7) demonstrated antigenic determinants both in the protein and the polysaccharide moieties of endotoxin isolated from autolysates of *Pseudomonas aeruginosa* Resently *Barber et al* (1, 2) have shown that antibodies to protein determinants are involved in the O agglutination of *Salmonella* strains

The endotoxin was separated by ultracentrifugation from a thermolabile precipitinogen but was still found to be contaminated with DNA which could have been present either as a DNA endotoxin complex or associated with other sedimentable material Both were thus excluded by the Sephadex G 200 column despite the apparent disaggregation of the endotoxin in the pH 12 buffer used for elution Further purification of the ultracentrifuged endotoxin was achieved both by treatment with phenol water and by block electrophoresis However treatment with phenol water destroyed the activity of *b* Purification by block electrophoresis is rather cumbersome for large scale preparation and involves exposure of the antigen to alkali and contamination by block material These methods were therefore not satisfactory as purification procedures

Digestion with DNase followed by repeated washings was the most convenient method for removal of nucleic acid constituents without resorting to drastic or complicated treatment of the antigen. It is difficult to establish the extent of purification. In procedures designed to check the homogeneity of the purified preparation the inhomogeneity of the endotoxin itself has to be considered. However, agar precipitation, paper electrophoresis and gel filtration failed to reveal serologically active or inactive impurities with the exception of small amounts of DNA.

The chemical data presented in this paper show that the aqueous ether endotoxin is a lipopolysaccharide protein complex with a major protein component. On a weight basis the serological activity of the carbohydrate component was far greater than the activity of the protein moiety. Dilutions of the purified endotoxin corresponding to 2·7 µg of protein were equivalent to 8 units of antibodies to the protein determinant, whereas dilutions corresponding to 0·1 µg of neutral sugar neutralized 8 units of antibodies to the polysaccharide determinant.

SUMMARY

The relationship between and purification of one polysaccharide and one protein antigenic determinant in the aqueous ether extract from gonococci has been studied. Separation of the components was not achieved by ultracentrifugation, treatment with aqueous phenol, electrophoresis or gel filtration. Antisera specific for each component precipitated both determinants. Presumably both determinants belong to the endotoxin complex of gonococci.

Treatment with DNase followed by repeated washings was the most satisfactory procedure for purification of the endotoxin.

The endotoxin was composed of protein, carbohydrate and lipid, protein being the major constituent.

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IMMUNOCHEMICAL CHARACTERIZATION OF AQUEOUS ETHER EXTRACTED ENDOTOXIN FROM *NEISSERIA GONORRHOEAE*

By

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Received 28.1.69

Previous investigations showed that the aqueous ether extract from gonococci contained one antigenic determinant of polysaccharide nature and one of protein nature, designated *a* and *b* respectively (21). Attempts were made to separate these components by several fractionation methods without success (22). Presumably both determinants belong to the aqueous ether endotoxin. Chemical analyses showed that the endotoxin was composed of lipid, carbohydrate and protein, the latter being the dominating component.

Further immunochemical analyses have been performed and this paper presents observations on 1) the qualitative and quantitative composition of the aqueous ether endotoxin, 2) the immunodominant sugars of the polysaccharide determinant, 3) the cellular origin of the endotoxin.

MATERIALS AND METHODS

N. gonorrhoeae strain 8551/64 was employed. Cultivation and harvesting of the bacteria and preparation of rabbit antisera to whole gonococci (anti G) have been described earlier (19).

Preparation of endotoxin. Extraction and purification of the aqueous ether endotoxin were carried out as described previously (21, 22). The purification procedure included treatment with DNase and washing with distilled water.

Preparation of cell walls. Nine grams of wet bacteria were disintegrated by passing the frozen bacteria thrice through a bacterial press (the X press). Isolation and purification of the walls were performed according to *Yoshida et al.* (39). The sucrose gradient centrifugation and the pile centrifugation were carried out at 1100 × g for 30 mins. The washed walls were lyophilized.

Analytical methods. Protein (14), *neutral sugar* (37), *lipid* (27, 29) and DNA (3) were determined as described before (2).

Nitrogen was determined by the micro Kjeldahl method (12).

Total phosphorus was determined by the method of *Fiske & Subbarow* (9) as modified by *Youngburg & Youngburg* (40).

Hexosamine was estimated by the method of *Rondle & Morgan* (26) with glucosamine as standard.

Heptose was sought by the cysteine sulphuric acid reaction of *Dische* (5) methyl-

pentose by the method of Dische & Shettles (6) and *uronic acid* by Dische's carbazole reaction (4)

Sialic acid was sought using the Ehrlich's reagent according to *Barry et al* (1) and the test for thiobarbituric acid reactive components was performed according to Warren (36)

Amino acids were estimated on a Beckmann 120 B amino acid analyser according to *Moore et al* (18) and *Spackman et al* (28) The substance was hydrolysed in 6 N HCl at 105 C for 18 hrs and the sample was dried *in vacuo* at 4 C over NaOH pellets

Paper chromatography Cell wall material was hydrolysed with 3 N HCl at 100 C for 3 hrs and the acid was removed by evaporation *in vacuo* at 4 C Hydrolysis of the endotoxin was performed with 2 N H_2SO_4 at 100 C for 3 hrs The acid was neutralized with barium hydroxide followed by centrifugation at 30 000 × g for 60 mins and lyophilization of the supernatant The sugars were then extracted with pyridine dried dissolved in distilled water and subjected to circular chromatography using Whatman No 1 paper

Solvent systems (A) n butanol acetic acid water (4 1 1) (13) and (B) ethyl acetate pyridine water (40 11 6) (8) Developing reagents Aniline hydrogen phthalate (24) or the silver nitrate reagent of *Trevelyan et al* (33)

Treatment with enzymes Treatments of the endotoxin with α glucosidase (Koch Light) β glucosidase (Sigma) and β galactosidase (Sigma) were carried out in 0 02 M phosphate buffers at pH 6 6 and 7 2 respectively The enzyme to substrate ratio varied from 1 5 to 1 50 Digestions were performed at 37 C for 20 hrs

Lyophilized cell walls were digested with trypsin (Trypure Novo) in phosphate buffered saline pH 7 4 at 37 C for 2 hrs The enzyme to substrate ratio was 1 50 After treatment with enzyme the mixture including controls were heated at 100 C for 5 mins and then dialysed against phosphate buffered saline

Serological methods Sensitization of sheep erythrocytes with the determinant *a* or *b* was performed using preparations of the aqueous ether endotoxin as described previously (2)

The indirect haemagglutination test and the test for inhibition of haemagglutination were performed as described in previous papers (19 21) The serological activity of antigen preparations was determined by the inhibition test using 8 agglutinating units (22) of the antiserum

Tests for inhibition of the indirect haemagglutination with commercial mono and oligo saccharides were performed as follows Each sugar was prepared as 0 28 and 0 56 M concentrations in distilled water *Twofold dilutions* of antisera were prepared in 0 1 ml volumes in buffered saline To each row of diluted serum was added 0 1 ml of the sugar solution After incubation at 37 C for one hour 0 1 ml of a 0 5 per cent suspension of sensitized erythrocytes was added Incubation and reading of the agglutination were performed as usual (19)

EXPERIMENTS AND RESULTS

Chemical Analyses

The quantitative chemical data are shown in Table 1 and Table 2 Whether based on the Folin value the total Kjeldahl nitrogen or total identifiable amino acids approximately 88 per cent of protein was found The preparation contained all the commonly occurring amino acids except cysteine cystine and diaminopimelic acid

The amounts of neutral sugar hexosamine and fatty acids were small none of them exceeding 3 per cent of the material Total phosphorus accounted for 0 35 per cent of the endotoxin and contaminating DNA one per cent In all the data account for about 95 per cent of the material

The sugars in the 3 N HCl hydrolysates of the endotoxin could not be identified This was probably due to interference by the large

amounts of protein breakdown products Hydrolysis with 2 N H_2SO_4 and extraction with pyridine gave good results provided all the pyridine-extractable material from at least 20 mg of hydrolysed endotoxin was applied to the paper

TABLE 1

Quantitative Analyses of Aqueous Ether Endotoxin from Neisseria gonorrhoeae *(Per Cent of Dry Weight)*

N	Protein	P	Neutral sugar	Hexosamine	Lipid	DNA
14 10	89	0 35	1 80	1 1?	3 00	1 00

The values listed are mean values of two or more determinations

TABLE 2

Amino Acid Composition of the Aqueous Ether Endotoxin

Amino compound	μ moles/100 mg	mg/100 mg
Lysine	47 8	7 0
Histidine	10 7	1 7
Ammonia	43 ,	0 7
Arginine	30 1	5 ?
Glucosamine	3 1	0 6
Aspartic Acid	?8 4	10 4
Threonine	33 (	4 0
Serine	33 ?	3 5
Glutamic Acid	81 6	12 0
Proline	?7	3 ?
Glycine	(0	4 9
Alanine	74 1	6 6
Valine	1 5	13 7
Methionine	? 3	0 1
Isoleucine	31 3	4 5
Leucine	5 (	7 3
Tyrosine	11 0	9 0
Phenylalanine	? ?	1 5
Totals	?44	87 6

Excluding glucosamine and ammonia
Not corrected for water uptake

Glucose galactose and glucosamine were detected in the solvent systems A and B Glucosamine (0 6 per cent of dry weight) was also detected on the amino acid analyser

The absorption curve of the products of the cysteine sulphuric acid reaction for heptose shows maxima at 390 mμ and at 50? mμ (Fig 1) The former maximum is due to hexoses whereas the latter suggests the presence of small amounts of heptose (?) So far no attempts have been made to identify the heptose

The thiobarbituric acid reaction gave no coloured product with unhydrolysed endotoxin After hydrolysis of 3 mg of endotoxin with 0 1 N H_2SO_4 at 80 C for 60 mins a small absorption peak was ac

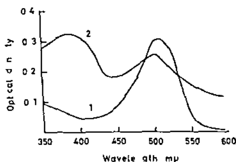


Fig 1

Absorption curves of the products of the cysteine sulphuric acid reaction of *Dische* 1 50 μg of D glycero – D galacto heptose 2 3 8 mg of aqueous ether endotoxin

hieved at 532 mμ This is the absorption maximum of deoxysugars (36) and probably originated from contamination with trace amounts of DNA No second peak appeared at 549 mμ the region for maximum absorption of sialic acid and 2 keto 3 deoxy octonate (KDO) The modified Ehrlich procedure for detection of neuraminic acid was negative Methylpentose and uronic acid were not detected

Inhibition of Haemagglutination with Mono and Oligosaccharides Effect of Enzyme Treatment

The results of inhibition experiments with 0 28 and 0 56 M concentrations of glucose galactose glucosamine and α and β lactose are seen from Table 3 Glucose and glucosamine did not inhibit the antibodies to the *a* determinant Inhibition with 0 28 M galactose resulted in moderate reduction of the titre of these antibodies The titre reduction occurred in repeated experiments and with antisera from different rabbits The inhibiting effect of α lactose was equal to that of β lactose both being considerably stronger than that of galactose The increase from 0 28 to 0 56 M concentrations of galactose and lactose resulted in a proportionate reduction of the titre

TABLE 3

Effect of Inhibition with Sugars on the Haemagglutination Titre of the Antibodies to Determinant a

		Inhibitor									
		D glucose		D galactose		D glucosamine		α lactose		β lactose	
	None	0 28 M	0 56 M	0 28 M	0 56 M	0 28 M	0 56 M	0 28 M	0 56 M	0 28 M	0 56 M
Titre of Anti Gc	512	512	512	128	64	512	512	32	16	32	16

Anti Gc Antiserum to whole gonococci

Galactosamine, rhamnose, lactulose, mannose, maltose, melibiose and stachyose did not reduce the titre of the antiserum. None of the sugars tested inhibited the agglutination of erythrocytes sensitized with determinant *b*.

The endotoxin was treated with various concentrations of β-galactosidase and α- and β-glucosidase. The serological activity of determinants *a* and *b* was not affected by the enzyme treatments. Digestion with α-galactosidase was not performed.

Relation of Determinants a *and* b *to the Cell Wall*

After density gradient centrifugation of the disrupted bacteria the same 3 layers as those described by *Yoshida et al.* (39) were recognized. The yield of cell wall material was very small: 1.5 mg per gram of wet bacteria. Considerable amounts of wall material had therefore been sedimented together with the unbroken cells in the sucrose gradient tube.

Whole cells were not seen in Gram-stained smears of the wall preparations. The diphenylamine reaction for DNA was negative. Paper chromatographic analysis of the 3 N HCl hydrolysate of 1 mg of wall material showed the presence of glucose, galactose and glucosamine, whereas ribose or other pentoses were not detected. The preparation was therefore probably free of nucleic acids. The small amount of material available did not allow further chemical analyses.

The lyophilized cell walls did not form homogeneous suspensions in buffers. After digestion with trypsin an opalescent homogeneous suspension suitable for serological work was obtained.

TABLE 4

Serological Activity of Cell Walls and of Aqueous Ether Endotoxin Determined by Inhibition of Haemagglutination

Preparation	MID with erythrocytes sensitized with	
	Determinant *a*	Determinant *b*
Cell wall	25	6.25
Endotoxin	6.25	3.10

MID: Minimal Inhibiting Dose in μg with 8 agglutinating units of the antiserum.

The trypsin-digested preparation inhibited the agglutination of erythrocytes sensitized with the *a* determinant or with the *b* determinant (Table 4). The wall material neutralized antibodies to the *b* determinant to a degree almost equal to that of the endotoxin, whereas the latter neutralized somewhat stronger antibodies to determinant *a*. After absorption of 1.6 ml of anti-Gc diluted 1:16 with 1 mg of wall material the antiserum no longer agglutinated erythrocytes sensitized

with the *a* or *b* determinant The titres of the unabsorbed antiserum were 512 and 1024 respectively

DISCUSSION

The present study has shown that the aqueous ether endotoxin originates from the cell wall of gonococci The difference in serological activity of the wall preparation and the endotoxin was small and the endotoxin therefore most probably constitutes a major cell wall component Previous investigations showed that the determinant group of the alkali extracted endotoxin was located superficially in the bacterial cell (20) The endotoxin therefore probably occupies the outer portion of the cell wall This agrees with the general concept that endotoxins form part of the cell wall (15) Moreover *Mergenhagen et al* (17) found that the *Veillonella* lipopolysaccharide originated from the outer cell membrane and it was recently shown by *Cesarini et al* (2) that the outer membrane in meningococci disappeared after extraction with aqueous ether The thermostability of determinants *a* and *b* (21) and their relation to the cell wall suggest that these determinants are identical to or part of the so called thermostable somatic antigen of gonococci (7)

Proteins accounted for nearly 90 per cent of the aqueous ether endotoxin whereas the values for each of the components carbohydrate and lipid did not exceed 3 per cent Investigations now in progress have shown that aqueous ether endotoxin from 2 other strains of gonococci have essentially the same composition Inasmuch as the constituents were found to be closely associated (22) aqueous ether endotoxin from gonococci are lipopolysaccharide protein complexes with a dominating protein component Most investigators working with endotoxin from Gram negative cocci have isolated the endotoxin by phenol water extraction Such endotoxins are lipopolysaccharides with a small peptide component *Tauber & Garson* (31) thus found that the lipopolysaccharide from gonococci contained only 0 3 per cent of Folin protein carbohydrate and lipid being the dominating constituents By contrast these same authors (30) found that the alkali extracted endotoxin from gonococci was a protein Their findings have largely been confirmed in our laboratory (*unpublished data*) The method of preparation therefore markedly affects the composition of endotoxic substances from gonococci as is also the case with endotoxins from enteric bacilli (10 23)

Ribi et al (25) introduced extraction of enteric bacilli with aqueous ether as a method giving biologically potent endotoxins *with a low* lipid content The occurrence of only 3 per cent of lipid in the gonococcal endotoxin was therefore not unexpected However aqueous ether endotoxins from the *Enterobacteriaceae as a rule* contain much

less protein and more carbohydrate than the endotoxin from gonococci

The aqueous ether endotoxin contained all the naturally occurring amino acids except cystine cysteine and diaminopimelic acid The same amino acids have been found in cell wall preparations from meningococci (35) and avirulent *Neisseria* species (11) The absence of diaminopimelic acid indicates that the mucopeptide was not present in the preparation examined

Glucose galactose glucosamine and heptose were found in the aqueous ether endotoxin Since the content of carbohydrate was very small the presence of other sugars in small amounts cannot be excluded Glucose galactose and glucosamine have been found by other investigators in lipopolysaccharides from gonococci (32) and meningococci (16) In addition to these same sugars *Yamakawa & Ueta* (38) found glycero mannoheptose in whole cell extracts from gonococci meningococci and avirulent *Neisseria* species Whether the occurrence of glucose galactose and glucosamine among several *Neisseria* species leads to serological cross reactivity of polysaccharide antigens from these bacteria is not yet known KDO was not detected in the endotoxin examined Nearly all endotoxins from aerobic Gram negative bacilli examined so far contain both KDO and heptose (for references see 15)

Galactose and lactose inhibited the agglutination of erythrocytes sensitized with the *a* determinant but not of erythrocytes sensitized with the *b* determinant Galactose thus seems to be the immunodominant sugar of the polysaccharide determinant group (or groups) Immunodominant sugars usually but not invariably hold a nonreducing terminal position *Uchida et al* (34) have shown that mannose the immunodominant sugar of *Salmonella* O factor 3 is nonterminal The lack of inhibition of glucose and glucosamine does not exclude the possibility that these sugars are part of the *a* determinant The inhibiting effect of lactose was stronger than that of galactose α and β lactose (α and β galactosyl (1–4) glucose) had equally strong inhibiting effects whereas melibiose (α galactosyl (1–6) glucose) did not inhibit the agglutination It is therefore probable that the anomeric configuration of the galactosyl residue does not influence the specificity but that this in part is determined by the position of linkage Although it is probable that there is a terminal galactose in the *a* determinant (1–4) linked to the next sugar further investigations are needed to elucidate the structure of the polysaccharide determinant

SUMMARY

The aqueous ether endotoxin from gonococci contained nearly 90 per cent of protein and about 3 per cent of each of the constituents carbohydrate and lipid The sugars found were glucose galactose gluco-

samine and heptose Among the 16 amino acids detected lysine arginine aspartic acid glutamic acid alanine valine and leucine occurred in the largest amounts

Galactose and lactose inhibited the reaction of antibodies to the polysaccharide determinant

Both the polysaccharide and the protein determinant were found to be components of the cell wall

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BRIEF REPORTS

SARCOIDOSIS

An in vitro Kveim Reaction Based on the Leucocyte Migration Test

By *Finn Hardt* & *John Wanstrup*

Due to international cooperation and standardization the intracutaneous Kveim test has proved useful in the diagnosis of the complex syndrome termed sarcoidosis (*Siltzbach* 1967)

This Kveim test however when performed according to traditional technique possesses conspicuous drawbacks in that 1) the test is time consuming (4 to 10 weeks) 2) the interpretation of the histological changes in the biopsy is often difficult and may be inconclusive 3) as the technique implies an introduction of antigen into the organism the test may alter the specific reactivity of which an estimation is desired Repeated examination will accordingly be of very limited value

For these reasons an *in vitro* test has always been desirable but *in vitro* assays for detection of cellular hypersensitivity in man have so far not been available

In vitro systems for an estimation of specific immunological reactivity of lymphocytes depending upon their ability to transform into blast cells in the presence of antigen have been widely used and the method was employed by *Hirschhorn et al* 1964 in sarcoidosis using Kveim antigen as the provoking agent

Although these results proved promising inasmuch as sarcoid lymphocytes responded positively little progress has been achieved in this field This may be due to methodological difficulties as the technique necessitates maintenance of lymphocytes in culture for 6 to 8 days Further it is probable that the antigen induced blast transformation of lymphocytes is an *in vitro* indication not only of cellular hypersensitivity but of humoral hypersensitivity as well

The antigen induced inhibition of the *in vitro* migration of variously derived immunocompetent cells has been used in several experiments since 1932 (*Rich & Lewis* 1932 *George & Vaughan* 1962 *David et al* 1964) and seems to present more exclusively an *in vitro* parameter of cellular hypersensitivity The principle of this reaction has recently been adapted for use also in man (*Søborg & Bendixen* 1967) and has been successfully applied in various clinical hypersensitivity states So far the leucocyte migration technique (LMT) which has been described in detail elsewhere (*Bendixen & Søborg* 1969) appears to be an *in vitro* parameter of cellular hypersensitivity uncorrelated to possible coexisting humoral hypersensitivity

The present study reports the preliminary results of this test used in a sarcoidosis—Kveim antigen system

Material and Methods

The case material comprises 7 patients with sarcoidosis newly verified by biopsy in various stages of disease activity as judged by the degree of fibro hyalinosis and 6 patients with active tuberculous infection The control group comprises 9 healthy persons

Peripheral leucocytes are isolated and placed in capillary tubes The tubes are then placed in culture chambers containing culture medium 150 µg of Kveim antigen (measured by protein content) is added to half the number of chambers As the Kveim antigen is preserved with phenol, which is toxic to leucocytes the Kveim suspension is dialysed against Hank's balanced salt solution for 48 hours

Received 14 69 from Medical Department A Rigshospitalet (Head K Brochner Mortensen) and Institute of Pathological Anatomy (Head G Teilum) University of Copenhagen Denmark

We are indebted to Dr T H Hurley Royal Melbourne Hospital Australia who generously supplied the Kveim's reagent.

MIGRATION INDEX

1.2

1.0

0.8

0.6

0

SARCOIDOSIS

TUBERCULOSIS

HEALTHY CONTROLS

Fig 1

The migration areas are measured 24 hours later by paper planimetry Migration index (MI) is calculated in the following way

$$\frac{\text{Migration in chambers containing antigen}}{\text{Migration in chambers without antigen}}$$

Thus MI principally indicates an inhibition of the cell migration if these values are lower than 1 0 + and a stimulation provided values greater than unity

Results and Comments

As apparent from Fig 1 all of the leucocytes derived from sarcoid patients showed considerabl inhibition of their migration *in vitro* when incubated with the Kveim antigen In contrast the control cultures from healthy or tuberculous persons showed no or only very slight inhibition

These results prove to us the diagnostic relevance of this *in vitro* Kveim system as leucocytes from sarcoid patients invariably showed pronounced inhibition Thus it was possible in 24 hours to obtain probably the same information as from the in vivo intracutaneous Kveim test which demands 6 to 10 weeks

We are aware that larger materials are necessary and that a comparative *in vivo—in vitro* Kveim study is desirable before definite conclusions can be drawn regarding the possible clinical importance of this LMT Kveim reaction

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Acta path microbiol scand 76 495–496 1969

ORAL CONTRACEPTIVES AND CERVICAL CANCER

A Preliminary Report of an Experimental Study

By *Eivind Myhre & Knut Bjoro*

The experiments were undertaken to investigate the effect of various hormonal influences and of antifertility compounds on chemically induced carcinoma of the cervix uteri in mice The complete report will appear in Norwegian Monographs on Medical Science Universitetsforlaget Oslo

Material and Methods

More than 400 mice of the WLO strain were employed in two main series one long term observation of about 30 weeks and a short term observation of about 15 weeks Cervix uteri of all animals were painted with a 1 per cent solution of 9 10 dimethyl 1 2 benzanthracene (DMBA) in acetone once weekly throughout the observation time Each main series was divided in groups of about 20 mice which were exposed to different treatments The hormones and the synthetic hormone acting compounds were administrated by subcutaneous implantation of pellets containing 40 per cent of the active agent and 60 per cent cholesterol The active agents in the pellets were progesterone 25 mg testosterone proprionate 10 mg chlormadinone acetate 5 mg quingestanol acetate 2 5 mg norethisteron acetate 5 mg and ethynyloestradiol in 10 5 2 5 and 1 5 mg pellets One group was castrated The control animals were only painted with DMBA —All animals were autopsied and several sections of uterus and vagina were thoroughly examined microscopically

Results

The *long term observation series* revealed 18 (94 7 per cent) cervical carcinomas in the 19 control animals all 11 castrated animals developed carcinomas as did the 19 chlormadinone treated animals 20 (90 9 per cent) of the 22 quingestanol treated mice displayed carcinomas 16 (84 2 per cent) of 19 mice in the norethisterone treated group had carcinomas only 9 (50 per cent) of 18 progesterone treated animal developed carcinomas and only 6 (31 6 per cent) of 19 animals in the testosterone treated group The ethynyl oestradiol treated (10 mg) group included more than 20 mice but the dosage was appearantly too high as most animals died some days or a few weeks following implantation of the pellets Only two mice lived longer in a miserable condition and had to be killed at 27 weeks No tumour was found

The *short term observation series* disclosed 1 (2 4 per cent) carcinoma in the 41 control animals 1 (9 1 per cent) in the 11 castrated animals and 1 (4 6 per cent) in the 22 chlormadinone treated *animals* No carcinomas were observed *in the* groups treated with quingestanol norethisterone progesterone or testosterone (each group comprising 18 to 23 animals) – The *ethynyl* oestradiol treated group was started with 5 mg in 25 animals but only 5 survived more than 5 weeks (average 7 5 weeks) and 3 of them had cervical carcinoma The dose of ethynyl oestradiol was reduced to 2 5 mg in another group of 24 animals Eleven mice lived longer than 5 weeks (average 8 7 weeks) and 5 developed carcinoma A still smaller dose of 1 25 mg of ethynyl-oestradiol was given to 20 animals Eight mice survived more than 5 weeks (average 11 1 weeks) and 4 of them had carcinoma A total number

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of 21 mice with varying doses of ethynyl oestradiol survived more than 5 weeks (average 9 3 weeks) and 12 (50 per cent) of them developed carcinomas

Discussion

The most important result of these experiments is that ethynyl oestradiol acted as a strong promotor of the DMBA induced carcinogenesis in mice as judged by the higher yield of carcinomas and the shorter induction time compared with the control animals Thus this synthetic oestrogen produced 50 per cent cervical carcinomas in a little more than 9 weeks whereas only 2 4 per cent of the control mice developed carcinoma in 15 weeks

Another important result is seen in the long term observation series where the progesterone treated animals developed carcinoma in only 50 per cent of the animals while the other groups including the control group developed 100 or rather close to 100 per cent carcinomas except the testosterone treated group

All oral contraceptives applied in the combination and the sequential pills consist of an oestrogenic and a progestational component The oestrogenic component in most if not all oral contraceptives is ethynyl oestradiol or its 3 methyl ether (mestranol) The results of the present experiments indicate that the composition of the oral contraceptives should be altered i e ethynyl oestradiol should be omitted The doses of ethynyl oestradiol and mestranol for oral contraception have true enough gradually been reduced but it seems now to be about time for the complete omission of these oestrogenic compounds Obviously species differences do exist but hormonal influence on carcinogenesis may be of general biological consequence

Conclusion

The composition of oral contraceptives ought to be guided in the direction of using progestational compounds only Our experiments indicate that the exclusion of the synthetic oestrogenic compounds will possibly reduce the existing fear of cancer development Further the potential anticarcinogenic effect of progestational substances is supported by our study

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MICRO X RAY DIFFRACTION OF CARTILAGE BIOPSY SPECIMENS IN ARTICULAR CHONDROCALCINOSIS

By

ANDERS BJELLE and BENGT SUNDSTROM

Received 7 ii 69

Articular chondrocalcinosis was first described as a clinical entity by *Zitnan & Sitaj* in 1958 (for reference see *Zitnan & Sitaj* 1963) Working on crystal identification in gouty arthritis McCarty and collaborators (*McCarty et al* 1962 *Kohn et al* 1962) accidently found atypical crystals in the synovial fluid of some patients The crystals were identified as calcium pyrophosphate dihydrate The syndrome was called pseudogout but further studies revealed that it probably was the same disease entity as the earlier described articular chondrocalcinosis During the last years an increasing number of cases has been reported (for reviews see *McCarty* 1966 and *Lagergren & Olhagen* 1967)

The present communication describes a diagnostic technique based on micro x ray diffraction of cartilage biopsy specimens

MATERIAL AND METHODS

Small cartilage biopsy specimens measuring approximately 0 1 cm³ have been removed from the dorsal part of the medial femoral condyle according to *Brighton* (1967) After a brief fixation in phosphate buffered formaldehyd thin sections—around 200 µ—were prepared from the unembedded material by sawing according to *Jansen* (1950) X ray diffractograms of calcification sites in the dried sections were obtained with Ni filtered Cu radiation in a Chesley camera (*Chesley* 1947) By generating the x rays at 40 kV/20 mA a photographic density for visual measurements of the reflexions is usually obtained at 15–20 hours exposure

RESULTS

The results of the above procedures are illustrated below in a patient in whom micro x ray diffraction analysis applied to aspirated synovial fluid as well as to surface scrapings of the affected cartilage were negative Very few crystals were seen in the synovial fluid by polarized light microscopy

Fig 1 shows a microradiograph of a sawn section where two areas of pathological calcifications are seen in the middle zone of the cartilage The marked mineral content in the lower half of the figure represents



Figs 1 3

Fig 1 Microradiograph of 200 μ thick cartilage specimen Two areas of pathological calcification are seen in the middle part of the figure The lower white area represents bone tissue and the tiny deposits at the upper left (arrow) are close to the articular surface × 28

Fig 2 X ray fiber pattern obtained from the larger (right) area of pathological calcification seen in Fig 1 The pattern is that of calcium pyrophosphate dihydrate × 1 5

Fig 3 Electron micrograph of a non decalcified ultrathin section of the calcium pyrophosphate dihydrate deposit Crystal size and orientation is variable and cannot be accurately estimated in two dimensional sections The occurrence however of comparatively large crystals > 4 μ in length is noted × 8 000

the normal bone and the tiny deposits seen at the upper part (arrow) are close to the articular surface Both areas of pathological calcification were easily seen macroscopically as delicate white non translucent areas in the sawn specimen and the larger one was positioned directly in front of the collimator

Fig 2 shows the obtained x ray pattern It is evident that the deposited crystals show no preferred orientation within the volume investigated This finding was also evident in diamond knife cut ultra thin sections as viewed by electron microscopy (Fig 3) When calculated the crystallographic spacings (d values in Ångstrom units) as well as the visually evaluated intensity of the x ray reflexions justify an unequivocal identification of the calcified deposits as crystals of calcium pyrophosphatedihydrate (for d values see *McCarty* 1966)

DISCUSSION

According to *McCarty and collaborators* the diagnosis of definite articular chondrocalcinosis should include demonstration of the specific crystals by definitive means e g characteristic finger print by x ray diffraction pattern (*McCarty* 1963) This analysis has generally been carried out by macrotechniques (*Debye Scherrer* cameras) on crystal preparations from synovial fluids In a revised classification *McCarty* (1966) considered the diagnosis as definite also when crystals compatible with calciumpyrophosphatedihydrate are demonstrated by compensated polarized light microscopy *and* typical calcifications are seen on conventional roentgenograms of the joints The demonstration of typical crystals by polarized light microscopy is often difficult especially in synovial fluids from patients with chronically symptomatic joints (*McCarty* 1966) The present diagnostic technique has enabled a definite diagnosis in cases where previously described diagnostic procedures have failed

To further elucidate the pathogenesis of the disease the demonstration of the crystals by definitive means for diagnosis is of particular importance The present technique allows additional histological investigations e g the relationship between the crystals and the surrounding cartilage matrix can subsequently be made on further sections from the same biopsy specimen

SUMMARY

A method for the diagnosis of articular chondrocalcinosis by way of micro x ray diffraction patterns from cartilage biopsy specimens is described The technique requires a minimum amount of crystal deposit it is comparatively rapid and enables further ultrastructural investigations of the crystals *in situ* in the cartilage matrix

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DIMENSIONS OF SOME WALL COMPONENTS OF SMALL ARTERIES AND ARTERIOLES IN THE NORMAL HUMAN KIDNEY

By

FINN JØRGENSEN and AGE CHR THOMSEN

Received 21 i 69

In a previous paper (1) we described the qualitative electron microscopic conditions in the small arteries in renal biopsy specimens from *individuals* without renal diseases On the basis of the same material the result of the measurements of various components in the *arterial* walls are now described Admittedly the number of *measurements* presented here is small and this is due to the fact that primarily the material was used for measurement of the glomerular basement membranes (2) However only a limited number of *normal* materials comprising renal biopsy tissue has been *published* and to our knowledge no ultra structural measurements have *been made* on normal human renal vessels Therefore we found it justifiable *to* publish the present results

MATERIAL AND METHODS

The procurement of the material *and* the criteria of normality have been described previously (1) *The material* comprised renal biopsy specimens from *five* women and two men aged between 33 and *76 years* None of the patients presented symptoms of renal diseases or diabetes mellitus Light microscopic studies of the biopsy specimens only *revealed* changes caused by ageing

Tissue for electron *microscopic* studies was fixed in OsO_4 dehydrated *in* acetone and embedded in Vestopal W *The* vessels were identified by light microscopy of 1 to 2 µm sections stained with Wright Giemsa or methyl *violet. Ultrathin* sections were cut with a *glass knife* on an LKB ultratome The sections were collected on copper grids and *contrasted* by means of uranyl acetate The microscopic studies were carried out using a Philips EM 100 B Photomicrographs were made at an initial magnification of 1300–7500 times and were then *magnified* photographically 10 time

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We are indebted to Mrs *J Barslund* laboratory technician for valuable assistance


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Definition of Type of Arteries

As described in a previous paper (1) it was possible by means of electron microscopic studies to classify into three types the arterioles and the smallest arteries present in the available renal biopsy specimens

(1) Small arteries and the first part of the vas afferens (Fig 1) All these vessels have a distinct lamina elastica interna The tunica media comprises from one to six layers of smooth muscle cells of the common type Measurements of all these vessels are collected in one group irrespective of the thickness of the vascular wall

(2) The juxtaglomerular part of the vas afferens (Fig 2) The vessels are recognized by the high content of granules in the tunica media and by the epitheloid character of their cells and the absence of an actual lamina elastica interna Close to the glomerular hilum irregular deposits of basement membrane like material are seen Because of the extremely varying conditions in the vicinity of the vascular pole no measurements of this type of vessels were made in the present study

(3) Vas efferens (Fig 3) The efferent arteriole has no actual lamina elastica interna On the other hand one or more subendothelial laminae are seen consisting of the basement membranes from the endothelial cells and the underlying muscle cells and the intervening ground substances The muscle cells are irregularly branched and they contain few granules The content of myofilaments is higher than in the vas afferens Measurements of all arteries within this group have been described collectively

Performance of Measurements

The measurements were taken from the photographically magnified pictures by means of a ruler at an accuracy of about 0 5 mm For each series of measurements the values found were then converted to the magnifications 13000 or 24300 times The results of the measurements from specimens of arteries of the same type were pooled from all the persons examined and treated statistically and then the values were converted into μm

Definition of Objects of Measurement (Fig 1 3 4)

The following measurements were taken

(1) Thickness of the vascular wall From the luminal plasma membrane of the endothelium where the endothelium was lowest to the peripheral basement membrane of the tunica media measured along a radius

(2) Thickness of the endothelium From the luminal to the basal plasma membrane The measurements were taken partly where the endothelium was lowest partly

(3) on a level with the centre of the nucleus

(4) Thickness of the endothelial basement membrane This membrane was often poorly delimited towards the periphery and sometimes it could not be distinguished from the remaining parts of the lamina elastica interna Measurements were taken in areas where the membrane was thinnest and most homogenous.

(5) Thickness of the lamina elastica interna From the basal plasma membrane of the endothelial cell to the luminal plasma membrane of the nearest muscle cell in areas where these structures ran parallel and where lamina presented a regular structure The lamina elastica interna was measured only in areas where it had its minimal values (with the exception of points of contact between muscle cells and endothelial cells)

Fig 1

Part of small artery showing the layers of the wall In the lumen (AL) finely flocculated precipitate of plasma proteins Endothelial cells (En) project into the lumen The internal elastic lamina (IEL) and the tunica media with its muscle cells (MC) are seen The muscle cells are surrounded by basement membrane (B) The adventitia contains collagen fibrils (Co) N nucleus Single arrow thickness of the vascular wall Dotted arrow thickness of the endothelium on a level with the nucleus Thick double arrow thickness of the muscle cell on a level with the nucleus Thin double arrow distance between the muscle cells (13000 ×)



Fig 2

Juxtaglomerular part of vas afferens The wall of the vessels consists of endothelium (En) and modified smooth muscle cells (MC) There is no real lamina elastica interna In tunica media numerous typical granules (G) AL arteriolar lumen *B* basement membrane N nucleus er rough surfaced endoplasmic reticulum (15800 ×)

(6) The thickness of the smooth mus le cells was measured on a level with the centre of the nucleus

(7) The thickness of the nucleus of the muscle cell was measured at the same site

(8) The distance betwe n the muscle cells Measured between cells which were regular and running parallel in areas where two independent or one common basement membrane was found between the cells On a level with points of contact no measurements were tal en

In all cases the object was to take measurements of purely longitudinal or t ansverse sections of the vessels assessed on the basis of course of structure appearance of the vascular lumen and the outline of the plasma membrane

In order to examine a possible mutual relationship the alues of wall thickness the thickness of the lamina elastica interna and the distance between the muscle cells were recorded in the individual vessels in some cases For this purpose only photographs in which all three structures were visible could be used Afterwards the three sets of measurements from a number of photographs were correlated



Fig 3

Section of the wall of the vas afferens Endothelium (En) and *modified smooth muscle cells* (MC) are shown No actual lamina elastica interna can be seen The muscle cells contain no granules or only very few AL arteriolar lumen B basement membrane (13000 X)



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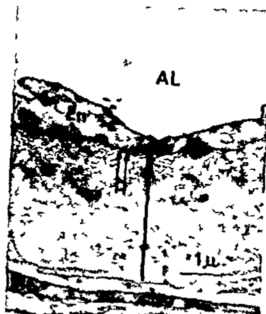


Fig 4
Part of an arteriolar wall with endothelium (En) and lamina elastica interna (arrow) The *endothelial basement* membrane (double arrows) is clearly demonstrated AL arteriolar lumen. MC muscle cell. (13000 X)

RESULTS

A survey of the results of the measurements is presented in Table I which shows the number of individual measurements means standard deviation and range as regards each individual object of measurement

The number of measurements depended on the available photographic material and varied considerably from one object to another

Also the range was wide as regards all the structures measured and in no case was the coefficient of variation below about 25 per cent

It appears from the table that the afferent vessels differ from the vas efferens by having (1) thicker wall (2) thicker lamina elastica interna (3) thicker subendothelial basement membrane and (4) greater and more varying distances between the muscle cells

Apart from an extremely varying height of endothelium the vas efferens shows a more homogenous structure than the vas afferens

The relationship between wall thickness of the afferent arteries and other structures was studied in 9 arteries There was a tendency for the lamina elastica interna to increase in width with increasing wall thickness but the increase was not significant ($r = 0.54$ $P > 0.1$) On the other hand there was a significant increase in the distance between the muscle cells with increasing wall thickness ($r = 0.842$ $P < 0.01$)

DISCUSSION

Measurements of distances in a histological material will always be subject to considerable inaccuracy because of changes in the structure of the tissues caused by the preparation As regards vessels some

TABLE 1

Results of Measurements of Small Cortical Arteries in Renal Biopsy Specimens from 7 Normal Human Beings

	Small arteries and first part of the vas afferens					Vas efferens				
	N	range µm	x̄ µm§	SD µm†	CV per cent	N	range µm	x̄ µm	SD µm	CV per cent
Thickness of the wall	47	?9 15 0	8 1	2 7	33	12	1 9–4 8	3 3	0 95	?9
Smallest thickness of the endothelium	97	0 04 0 78	0 30	0 17	57	24	0 02–0 49	0 14	0 13	93
Thickness of the endothelium on a level with the nucleus	?8	1 6–5 4	3 4	1 0	?9	10	2 7–7 3	3 9	1 5	38
Thickness of the basement membrane of the endothelium	39	0 08–1 4	0 40	0 31	77	18	0 04–0 1?	0 08	0 02	25
Thickness of the lamina elastica interna as a whole	106	0 16–3 6	1 06	0 65	61	32	0 08–0 ?9	0 20	0 06	30
Thickness of the muscle cell on a level with the nucleus	25	1 8–5 5	4 0	0 95	24	7	1 4–3 [illegible]	2 8	0 67	24
Thickness of the muscle cell nucleus	31	1 2–3 6	2 3	0 61	27	6	0 8 ?8	1 9	0 69	36
Distance between the muscle cells	93	0 08 1 ?	0 28	0 20	71	22	0 08 0 31	0 16	0 06	38

Number of measurements
§ Arithmetic mean
† Standard deviation
Coefficient of variation

changes in the degree of contraction may also occur after removing the vessels from the tissue This will often be of the nature of wrinkling of the vascular wall Some of these disadvantages may be overcome in animal experiments e g through fixation by perfusion but they can not be avoided in a human material of renal biopsy specimens

It is a further disadvantage that it may be difficult by electron microscopic studies to identify a certain vessel and to decide whether it is a lobular artery or the first part of the afferent arteriole Since our material represented vessels of the same dimensions and of the type which is seen most often in renal biopsy specimens we decided to pool the results from the two types of arteries Arcuate arteries are not included in the study

The third difficulty is to ensure that the vascular structure examined is not cut obliquely but transversely or longitudinally This cannot be carried out with certainty and therefore thickness measurements as a rule will provide too high average values Statistical analysis of a sufficient material orientated and cut at random will provide information as to a probable average value but for this purpose a large number of measurements and uniform morphological structures are required The method is suitable for mitochondrial measurements (4) but the present material is too small and too inhomogenous for such analyses

In order to assess any possible changes in a pathological material (3) we had therefore to treat the normal material and the kidneys presenting pathological changes in exactly the same way both as regards the histological technique and the actual measurements The absolute values found have a bearing upon these conditions only and it must be expected that somewhat different values will be obtained when a different histological technique is employed (e g using another embedding agent)

It is also probable that measurements of vessels of greater dimensions (both in the kidney and in other regions) may give different values as regards the thickness of the lamina elastica interna and other measurements

The applicability of the normal material is supported by the fact that we succeeded in demonstrating significant differences between this material and a corresponding material comprising diabetic patients (3)

SUMMARY

Electron microscopic studies of biopsy specimens from the renal vessels were undertaken in seven adults without renal diseases The thickness of the vascular wall the endothelium the basement membrane of the endothelium the lamina elastica interna the muscle cell on a level with the nucleus the nucleus of the muscle cell and the distance between the muscle cells were measured on the electron

microscopic photographs The measurements were taken in two groups of vessels comprising (1) the lobular artery and the first part of the vas afferens and (2) the vas efferens The values found are tabulated showing means and standard deviations

The material will serve as reference material for a study of corresponding vessels in diabetic patients

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PULMONARY BONE MARROW EMBOLISM FOLLOWING EXTERNAL CARDIAC MASSAGE

By

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Received 8 II 69

Pulmonary bone marrow emboli were first described by *Lubarsch* in 1898 Since then strong emphasis has been placed on the role of trauma in producing bone marrow emboli *Rappaport* (1951) pointed out that fractures resulting in marrow emboli are not necessarily due to external injury but may result from sudden muscular contraction as they occur in convulsions from a variety of causes such as accidental electrocution generalized convulsions after eclampsia tetanus and treatment for certain mental disorders Two cases of bone marrow embolism following sternal puncture have been described (*Joell* 1959) With the publication of *Kouwenhoven's* work (1960) on a simple method of external cardiac massage a new aetiologic factor arose Recently *Paaske et al* (1968) writing on the complications of external cardiac massage in forensic cases found only one example of pulmonary bone marrow emboli among a total of 268 patients who prior to death had received external cardiac massage Since our experience was considerably different a survey of the histological sections of the lungs of all patients in the autopsy material at Louisville General Hospital between January 1 and December 31 1967 was performed on patients who had external cardiac massage prior to death

MATERIAL

Among the 510 patients who were autopsied that year 58 were found to have had external cardiac massage prior to death The information concerning external cardiac massage was taken from the medical record Seven out of these 58 were under the age of four months and were excluded none had bone marrow emboli On histological examination of the remaining 51 patients (22 females and 29 males with an average age of 59 and a range from 16 to 90 years) eight cases were found with pulmonary bone marrow emboli The emboli were not noticed on gross examination in any of the cases Pulmonary bone marrow emboli were defined as clusters of haematopoietic cells especially megakaryocytes eosinophilic myelocytes and normoblasts together with fat cells all within a vascular lumen with or without bone fragments (Fig 1) The average number of slides per case was five both in the total group and in the eight cases

Table 1 shows an even distribution between the sexes in the group with pulmonary bone marrow emboli The average age was 68 years ranging from 60 to 81

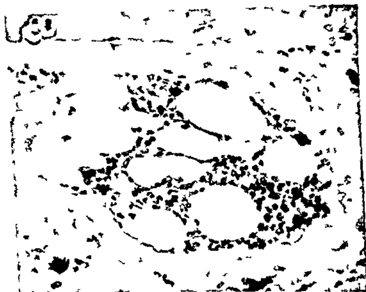


Fig 1

hotomicrograph of pulmonary bone marrow embolus completely occluding the vascular lumen The myeloid elements show active haematopoiesis (Haematoxylin and eosin × 400)

years The cause of death and underlying disease is indicated All eight patients received cardiac massage and oxygen In addition a few of the patients received intracardial adrenalin injections None of the eight patients had received cardiac massage before and none of the eight responded to the resuscitation attempts Only two of the eight patients were noted to have fractures of ribs sternum or vertebrae In none of the patients was there indication of other trauma In most of the cases the bone marrow emboli were multiple and on an average were shown in over half of the available lung slides The bone marrow emboli were found in vascular lumina from the smallest to those up to 250 microns in diameter In none of the cases was the pulmonary bone marrow embolism thought to have caused death In five of the cases, the bone marrow embolism was noted in the autopsy report In two patients there was coincidental pulmonary emboli No effort was made to determine the extent of fat embolism although several reports (*Ellis et al* 1968) have indicated that bone marrow intracellular fat is the usual source of embolic fat following trauma.

DISCUSSION

In this material eight out of 51 adult patients who received external cardiac massage prior to death developed pulmonary bone marrow embolism giving an incidence of 16 per cent This is in agreement with *Baringer et al* (1961) who gave an incidence of 13 per cent (6 out of 46 patients) also with *Bynum et al* (1963) who reported 12 per cent (6 out of 50 patients) It is in contrast to *Lane et al* (1965) who reported 62 per cent (10 out of 16 patients) and *Yanoff* (1963) who reported 91 per cent (10 out of 11 patients) These figures however must be interpreted with some reservation The incidence must of course vary with the group of patients and the technique used for

TABLE 1

Legends to Table 1

Case	Age	Sex	Number of slides with bone marrow emboli/Total number of slides	Fractures	Cause of death/ underlying disease
A67-10?	60	♀	4/7	No	Septicaemia/diabetes mellitus
A67-177	74	♂	3/6	No	Lobar pneumonia/adenocarcinoma of prostate with metastases to lung and kidney
A67-307	60	♀	1/6	No	Lobar pneumonia
A67-314	73	♂	4/7	No	Pulmonary congestion and oedema/congestive heart failure
A67-419	61	♀	2/2	Multiple bilateral rib fractures	Anasarca/diabetic heart and renal failure
A67-498	87	♂	2/4	No	Retention of bronchial secretions/incarcerated right inguinal hernia
A67-501	65	♀	2/6	Multiple bilateral rib fractures	Massive pulmonary embolus/mitral stenosis and insufficiency
A67-502	60	♂	3/4	No	Heart failure cardiac arrythmia/ chronic obstructive lung disease

external cardiac massage It is also noted that the incidence drops with an increasing number of patients

The mechanism by which the bone marrow comes to the lung during external cardiac massage is generally accepted to be through fractures in the sternum ribs or vertebrae By injection studies Pi olato (1944) has demonstrated that a communication exists between the sternal marrow cavity and the mammary vein This communication must have been known to clinicians who administered parenteral fluid via the sternal route in the early 1940 s The vertebral column is constructed in such a way that minimal trauma would result in a displacement of marrow fragments In the vertebral bodies a large sinusoid plexus is situated with haematopoietic tissue separated from the sinusoids by only a single cell limiting membrane

It is surprising in our group of patients to note how few have frac

tures of either sternum ribs or vertebrae It is easy to see why fractures of the vertebrae would be missed during an autopsy There may be several explanations for the uninvolvement of the ribs the most likely being that the fracture is a microfracture without involvement of the cortex and therefore escapes gross recognition The fractures may also be noted by the prosector but disregarded since it was thought to be postmortem Pulmonary bone marrow embolism can occur very rapidly in these eight patients however it is impossible to know how soon the embolism took place They were given external cardiac massage for a period varying from 10 to 60 minutes

Ogata (1912) has been concerned with the ultimate fate of the pulmonary bone marrow emboli He injected a suspension of bone marrow fragments from rabbits into other members of the same litter and studied the lungs of the animals after varying intervals He noticed that the bone marrow fragments underwent gradual disintegration and finally were transformed into a calcified mass *Lubarsh* in 1905 arrived at some figures which are likely to resemble the situation in man He injected the animals with an emulsion of liver and kidney and produced emboli that originated from the animal s own bone marrow Bone marrow fragments were still recognizable as such after six days It took two weeks before a gradual disintegration occurred and four weeks for completion

In general it can be said that the presence of pulmonary bone marrow fragments regardless of origin has medico legal significance since they indicate an underlying bone fracture even if such was not found at autopsy The total significance of pulmonary bone marrow emboli in external cardiac massage has not yet been determined When found they may be an indication that the resuscitation measures were too vigorous

SUMMARY

Attention has been called to the frequent finding of pulmonary bone marrow embolism in patients who received external cardiac massage An incidence of 16 per cent 8 out of 51 adult patients was noted in the autopsy material for 1967 in Louisville General Hospital This finding is in agreement with several other reports on the subject The relationship between fractures of ribs sternum or vertebrae and pulmonary bone marrow emboli is emphasized

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LACK OF RESTORATION OF THYMIC TISSUE FOLLOWING PARTIAL SURGICAL THYMECTOMY IN THE MOUSE

By

KIRSTINE BORUM

Received 1 iii 69

The thymus is derived from the third branchial pouches of the mammalian embryo During development the thymic tissue moves anteriorly and the right and left lobe fuse in the midline One can consider the mammalian thymus as one organ with two lobes or as a paired organ In either case one might expect to find a proliferative response upon removal of one of the thymus lobes

It was the aim of the present work to study the weight changes over long periods of time in the intact lobe of the mouse thymus following the surgical removal of the other lobe in order to see whether early as well as late changes might occur

In mice of the CBA strain the thymus increases in size from birth until four to five weeks of age (*Borum* 1965) After six to seven weeks of age a continuous slow decrease in size the so called age involution takes place Because of these biological characteristics of the normal thymus mice of different ages were investigated

MATERIAL AND METHODS

94 litters of an inbred CBA strain *were used* As far as possible each litter was divided into an equal number of sham or non operated and partially thymectomized mice within each sex The thymus involute during pregnancy and lactation so the female mice had to be kept as virgin mice Four different ages were studied In Group 1 (including 53 males and 52 females) the mice were six days old at the moment of operation and possessed a rapidly growing thymus The mice of Group 2 (51 males and 44 females) were three weeks old at the moment of partial thym ectomy their thymus glands had already reached the maximum size relative to *body weight* but were still growing in absolute size Group 3 (40 males 47 females) consists of six weeks old mice with a large thymus in which growth has ceased and Group 4 (51 males and 40 females) comprised three months old mice whose thymus glands *were in the* process of age involution A total of 378 mice (195 males and 183 females) survived throughout the observation periods

The operation was performed in ether anaesthesia in the case of six days old mice all other mice were operated in Nembutal® anaesthesia The skin of the ven

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This investigation was supported by the *Swedish Cancer Society*

tral thorax was shaved and an incision made in the midline The sternum was cut to the level of the second costa The layer of the pretracheal muscles was split in the midline and the upper poles of the thymus lobes were thus exposed One of these was gently dissected free from the underlying tissue and the whole lobe was sucked out through a glass tube Immediately upon removal of the lobe the incision was closed with three silk sutures in the skin A thin film of Nobecutan® was sprayed upon the cicatrice Sham operation was performed in the same way except that no thymic tissue was removed The right and the left thymic lobe are not of exactly the same size therefore the right and the left thymic lobe was removed alternately

The animals were killed 1 2 3 4 5 and 10 weeks after the operation and their thymic tissue dissected out and weighed in pre weighed plastic tubes with lid Routine histological sections of thymus tissue from each experimental group were studied

In order to study the restorative response when more than 50 per cent of the thymic tissue was removed an additional experimental group of 39 (14 males and 25 females) two weeks old mice had an amount estimated to 3/4–9/10 of the whole thymus gland removed This operation also conveys upon the thymus lobe under study a lesion which might give a further stimulus to regenerative growth The mice were killed 3 5 and 10 weeks after the operation

RESULTS

As the size of the thymus gland is different in the four age groups under study and also shows differences between the two sexes it is necessary to present the results for each age group and for each sex separately As can be seen from Fig 1 the weight of the thymus lobe left in situ following the extirpation of the other lobe amounts in all cases to about half of the weight of the thymus gland in the sham operated or unoperated control animal irrespective of the age at which the operation was performed and irrespective of the length of the observation period The thymus tissue left in situ did not show any compensatory weight increase Apparently it grew and involuted according to the age of the animal as if no operation was done and the contra lateral lobe was still there

Table 1 shows the weight of the remaining thymic fragments 3 5 and 10 weeks after the extirpation of estimated 3/4–9/10 of the whole thymus gland from 2 weeks old mice The drastic reduction in the amount of thymic tissue together with the lesion encountered in the remaining thymus fragment do not seem to induce any compensatory weight increase in the remnant left in situ

Histological sections did not show any difference between thymus glands from lobectomized and from sham or non operated mice within each experimental group

The thymus weight of sham operated individuals and non operated individuals did not differ within the same age and sex

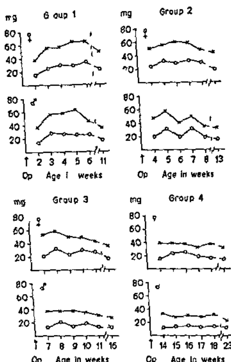


Fig 1

ight of thymic tissue in mice (a total of 378) at various times after surgical noval of one thymus lobe O——O and in sham or non operated control mice ——× The mice in Group 1 were six days old the mice in Group 2 were three eks old the mice in Group 3 were six weeks old and those in Group 4 were three onths old at the moment of operation Each point is the average value obtained from at least three mi e

TABLE 1

ight of Thymic Tissue in Mice (a Total of 39) 3 5 and 10 Weeks after the Surgi il Extirpation of Estimated 3/4–9/10 of the Whol Thymus Gland and in Sham r Non Operated Control mice The Number in ( ) Indicates the Number of Animals

Observation period	Males		Females	
	Partially thyme tomized	Sham or non operated	Partially thymectomized	Sham or non operated
3 weeks	1? mg (?)	62 mg (?)	11 mg (5)	74 mg (3)
5 weeks	9 mg (2)	40 mg (?)	?3 mg (?)	59 mg (2)
10 weeks	8 mg (?)	3? mg (4)	5 mg (8)	32 mg (5)

DISCUSSION

In contrast to the restoration of liver tissue after partial hepatectomy and the compensatory hypertrophy of the remaining kidney following unilateral nephrectomy the thymus gland shows no restorative or

compensatory response following partial surgical thymectomy The homoeostatic mechanisms governing that the amount of liver and kidney tissue respectively tends to be constant do apparently not function in the case of thymus

Another indication of the same thing is the *autonomous growth of* multiple thymus grafts in one animal reported by *Metcalf* (1963) Each implanted thymus grew and involuted independent of the presence of the other thymus glands though the experimental mice sometimes carried as many as fifty grafts *Hoskins* (1921) found no compensatory hypertrophy of the remaining thymus after partial thymectomy in the tadpole and implantation of a thymus gland to a recipient tadpole did not influence the size of the graft or the size of the autochtonous thymus

When however the amount of thymic tissue is reduced by means other than surgical extirpation e g treatment with corticosteroids or ionizing radiation a rapid weight increase is observed following the acute involution (*Ito & Hoshino* 1961 *Borum & Berglund* 1964 *Smith & Kieffer* 1957)

One difference between the drug or radiation induced involution and the surgical removal of part of the thymic tissue is that the former procedures extinct mostly lymphocytes in particular cortical lymphocytes while the latter one also involves removal of medullary tissue *Metcalf* (1963) has pointed out that the presence of medullary tissue is necessary for the successful take and growth of a thymic graft This suggests the significance of the thymic medulla for maintenance of the thymic cortex

Another difference is that the reticular framework and especially that of the cortex seems more or less intact following the treatment by corticosteroids and radiation but is removed by the surgical extirpation This framework might be a necessary prerequisite for the re population with lymphocytes of the experimentally involuted thymus gland

The two here mentioned possible explanations of the different regenerative response following partial surgical thymectomy and following corticosteroid or radiation induced involution do not *mutually* exclude each other It may be that a given amount of *medullary tissue* can support a certain amount of reticular framework and thereby a certain proportion of lymphocytes in the thymus Surgical removal of part of the thymus gland involves removal of proportional *parts* of framework and medulla and does not initiate a restorative *response*

One could question if the lobe or fragment of thymic tissue left in situ after surgical extirpation does function at a higher rate *even if* it does not increase in size *Metcalf* (1964) found no increase *in mitotic* activity in the remaining fragment during the first 48 hours *after* partial thymectomy It is also worth recalling that the mitotic rate and the cell production under normal conditions in the thymic gland *is indeed*

very high As showed by *Borum* (1968) the cell production in the thymic cortex amounts to the renewal of a mass of tissue corresponding to the whole cortex every 27th hour in the mouse

SUMMARY

378 mice of both sexes of an inbred CBA strain were used 182 of the animals were partially thymectomized i e had one thymus lobe removed and 196 were sham operated or non operated controls Four age groups were studied 1) six days old 2) three weeks old 3) six weeks old and 4) three months old at the moment of operation One two three four five and ten weeks after the operation the mice were killed and the remaining thymic tissue weighed The weight of the thymus remnant was always about half of that of the thymus of the control animal within the same sex— irrespective of the age at operation and the length of the observation period It is concluded that there is no regeneration of thymic tissue following partial surgical thymectomy the thymic tissue left in situ grows and involutes as under normal conditions uninfluenced by the removal of half of the original amount of thymic tissue In a small additional group of 39 two weeks old mice among which 21 had the estimated 3/4–9/10 of the thymus gland removed and 19 were sham or non operated controls no weight increase in the thymus remnant was found No histological changes between the thymus from control animals and from partially thymectomized animals were detected

The different behaviour of thymus tissue after partial surgical thymectomy in comparison with liver tissue after partial hepatectomy and kidney tissue after unilateral nephrectomy as well as the difference between partial surgical extirpation and the partial removal of thymic tissue by means of e g corticosteroids or ionizing radiation is discussed

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THE OCCURRENCE OF VIRUS PARTICLES IN ROUS HAMSTER SARCOMA

By

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It is well known that some strains of Rous virus are capable of inducing sarcomas in Syrian hamsters (*Ahlstrom & Forsby* 1962 *Klement & Svoboda* 1963 *Shevlyaghin* 1963 *Klement* 1964) A connection between the viral and the hamster cell genome is indicated by the successful back grafting of hamster sarcoma cells to chicken yielding a Rous virus producing sarcoma (*Ahlstrom & Forsby* 1962 *Vigier* 1966 1967) and by the finding of CF antigens in the hamster sarcoma cells (*Huebner et al* 1964)

So far no infectious Rous virus has regularly been isolated from the Rous virus induced hamster sarcomas (1 Ha sarcomas) but it has been reported that the sarcoma cell occasionally might be able to synthetisize infectious virus (*Svoboda & Klement* 1963 *Klement & Vesely* 1965)

In the electron microscope virus particles morphologically similar to the avian leukosis group have been found in the Rous dog sarcoma (*Rabotti et al* 1966) and recently in the Rous rat sarcoma cell in tissue culture (*Valentine & Bader* 1968)

In the present paper a report is given on the findings of virus particles in three different Rous hamster sarcoma lines *in vivo* Virus particles could also be demonstrated in the RHa sarcoma cell after it had been explanted and grown *in vitro*

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The skilful technical assistance of Miss *A Centerwall* Mrs *I S Hedström* Mrs *K Hillbert Nilsson* and Miss *B Nordstrom* is gratefully acknowledged

MATERIAL AND METHODS

Rous virus Strain Schmidt Ruppin (SR-RSV) was used The virus suspension was obtained from homogenates from chicken sarcomas purified by ultracentrifugation (*Moloney* 1960) The virus suspensions were pooled and kept in saline at —70 C. The virus titre tested at regular intervals according to *Temin & Rubin* (1958) was around 2-5 $\times$ 10^5 FFU/ml

Rous hamster sarcoma Tumours were induced by injection of 0 1 ml of the RSV suspension into new born hamsters (before the age of 4 days) The injection needle was passed through the muscles of the right thigh into the lower lumbar region After about 2 weeks a tumour appeared at the injection site and sometimes multiple tumours developed on the back attached to the skin The tumours rapidly increased in size became softer partly necrotic and often hemorrhagic The increasing growth of the tumours killed the animals 4–6 weeks after the inoculation Consistent with previous reports almost 100 per cent of the hamsters developed sarcomas (*Ahlstrom & Forsby* 1962)

Specimens from the injection site were excised on the first day after the injection of the virus and then on every second or third day until the appearance of tumours in a parallel series of hamsters Tumour tissue was then removed every third or fourth day for 3 weeks Specimens were also taken from a serially propagated tumour line induced in 1963 by SR-RSV and kept in continuous serial transfer since then Material was taken from passages no 62 95 114 1?4 and 13? Ultra structural examination was also done of serially transferred tumours from two other tumour lines which were induced for the present study vi passages no 1 2 3 and 13 in one line and the six first tumour generations in the other The latter tumour line was induced by SR RSV virus purified from sarcomas in a leukosis free strain of chickens

The presence of virus in RHa sarcoma cells tested on chickens and new born hamsters All RHa sarcomas have regularly been tested for the presence of the RSV genome Specimens from primary or serially transferred tumours were finely minced diluted with phosphate buffered saline 0 5 ml of a decanted cell suspension was injected into the wing web or the pectoral muscles of chickens aged 3–6 weeks

In parallel crude and purified cell free homogenates have been tested in an analogous way The homogenates were purified according to *Moloney* (1960) This technique allows a large amount of tumour tissue to be concentrated in a small volume of fluid after the final step Crude and purified homogenates have been prepared and tested from most of the RHa sarcomas which ultrastructurally contained evident virus particles

The same tests were also performed with cell suspensions from RHa sarcomas established in tissue cultures and from several sub ultures of the tumour cells in which virus synthesis was seen in the electron microscope Virus suspensions were obtained through homogenisation or sonication (see below) followed by centrifugation of the cell material In addition culture medium from serum bottles with virus producing cells was tested

Cell free preparations were injected in both chickens and hamsters in parallel and as controls parallel series of chick n and hamsters were injected with the riginal pool of SR RSV The injection in chickens was done in the wing web in liquots of 1–? ml and in the new born hamsters the virus suspensions were injected into the lower back through the thi h muscles in volumes between 0 ?–0 5 ml All test animals were observed during three months

T ssue Cultures

Rous hamster sa coma cultures Tumour material from passages no 12? and n 139 of the serially propagated RHa sarcoma was cultured in Ea le s minimal es ential me lium with 0.3 per cent trypto e phosphate 10 per cent calf serum and ant bioti s (penicillin 100 IU/ml, streptomycin 50 µg/ml) in 5 per cent CO atmos phere As a rule the tumour grew slowl in the beginning and the first subcultures coul I n t be obtained until after 10–1 days Gradually the tumour cells became adapte l and it was necessary to prepare subcultures every fourth or fifth day until the tum ur cells became less viable after 10–12 weeks

For electron microscopy the cell layer was tryp inized and a small pellet of

viable cells was obtained after centrifugation at 160 g of the resuspended RHa sarcoma cells

Sonication of cell suspensions was performed at 0 C using a MSE Ultrasonic power unit (18–20 kC/sec) during 10 × 30 sec

Chicken embryo fibroblasts Primary cultures of 9–10 days old leukosis free chicken embryo cells were prepared in serum bottles or 100 mm petri dishes (Falcon) and virus inoculations were made on secondary cultures according to *Temin & Rubin* (1958)

Hamster embryo fibroblasts Fibroblasts from 12–15 days old hamster embryos were cultured in the same way as described above All tests were performed on secondary cultures

In parallel serum bottles containing chicken fibroblasts and bottles with hamster fibroblasts were kept at +33 C. In addition both cell cultures were kept in a medium with sodium hydrocortisone added in conc of 0 1 1 0 and 10 0 μg/ml culture fluid both at 37 C and 33 C (Hydrocortisone sodium succinate U S P Upjohn Co Kalamazoo Michigan USA)

All subcultures which were inoculated with virus suspensions were maintained in continuous serial subcultures for 8–10 weeks It was as a rule possible to get enough viable cells in small pellets for electron microscopy as described above

Tests for viral activity 0 5 ml–2 0 ml of undiluted cell free preparations from RHa sarcomas were inoculated into the first subculture of chicken or hamster fibroblasts in serum bottles All inoculated subcultures were examined daily or every second day At regular intervals subcultures in petri dishes were prepared covered with agar and stained with Giemsa after a suitable interval

Electron Microscopy Small tumour fragments were as soon as possible fixed in 3 per cent glutaraldehyde in saline (pH adjusted to 7 2 with a few drops of phosphate buffered saline) at 4 C for about 24 hours After washings in phosphate buffered saline the specimens were post fixed in 3 33 per cent OsO_4 s collidine (*Wood & Luft* 1965) Osmium fixation was performed for 60 min at 4 C and was followed by repeated washings The specimens were dehydrated in graded ethanol and embedded in Epon 812 with propylene oxide as intermediate solvent (*Luft* 1961) Some specimens were embedded in Vestopal W (*Ryter & Kellenberger* 1958) and then graded acetone was used as a dehydration agent. As a rule it was necessary to enhance contrast by dissolving 1 per cent phosphotungstic acid in 70 per cent ethanol and allowing it to infiltrate the specimens for 60 min during the dehydration procedure Sectioning was performed on a LKB Ultrotome III and some grids were post stained in lead citrate according to *Venable & Coggeshall* (1965) All grids were coated with formvar films stabilized with carbon vapor The specimen grids were examined in a Zeiss EM 9 electron microscope equipped with a condensor Cell pellets obtained from the tissue cultures were treated in the same way as described above except that the initial fixation took place in 1 per cent glutaraldehyde in phosphate buffer (*Maunsbach* 1966) at +4 C for 2 hours

RESULTS

The Ultrastructure of the Rous Hamster Sarcoma Cell

The ultrastructural appearance of the Rous hamster sarcoma cell is shown in Figs 1–2 which were obtained from primary tumours In the early stage of the tumour development the sarcoma cells are separated by bundles of collagen but later they are surrounded by amorphous cell material probably debris from dead or necrotic cells In addition red blood cells are found in increasing amounts There is a prominent interstitial oedema which widely separates the tumour cell Many of the sarcoma cells show evident signs of severe damage such as disintegration of outer cell membranes swelling and fragmentation of mitochondria as well as dissociation of the nuclear membranes (Figs 3–4)

Two types of cells can be distinguished As a rule the primary sar

coma is dominated by irregular large cells with several long and slender cytoplasmic processes intermingled with neighbour cells The cells always show a well developed endoplasmic reticulum coated with ribosomes In several cells a loose fibrillar matrix can be found in the central part of the cytoplasm One or several Golgi systems can usually be seen There is always a large amount of free ribosomes or polyribosomes in the cytoplasm of the viable cell The nucleus is huge with a finely granular evenly dispersed chromatin and as a rule only a very small brim of condensed chromatin is found along the rather distinct sometimes folded nuclear membrane This type of cell is demonstrated in Fig 2 The other cell (Fig 1) is generally smaller but forms even more irregular cytoplasmic processes In the cytoplasm there is a sparse endoplasmic reticulum and only a few mitochondria can be seen The most prominent feature of this cell is the proportionally large nucleus somewhat irregularly outlined with heavy condensation of chromatin along the nuclear membranes

The RHa sarcoma cell established *in vitro* generally has the ultrastructure of the first type described above and virtually no cells resembling the other type have been observed The cell membrane is coated with several microvilli The cytoplasm is fairly rich moderately electron dense and contains several large mitochondria and an abundant endoplasmic reticulum sometimes dilated and forming vesicles There is also several primary and secondary lysosomes in the cytoplasm containing ingested material from the culture medium A Golgi apparatus is usually found as well as the central fibrillar matrix described above The latter is even more prominent in the cultured cells and is especially prominent in cells which show signs of minor damage Characteristically all mitochondria secondary lysosomes and larger vesicles as well as the endoplasmic reticulum are distributed peripherally and outside this fibrillar zone (Fig 9)

As in the primary tumours several of the cultured cells were found to be damaged

The Finding of Virus Particles

Virus particles were found in cells from the primary RHa sarcomas as well as in the serially transferred sarcomas In 5 out of the 15 primary sarcomas virus particles were observed and budding of virus particles into intracytoplasmic vesicles could be demonstrated (Fig 8) All virus particles were very uniform and showed an outer diameter averaging 75 mμ Particles not in contact with cell membranes showed a morphologic appearance akin to that of the viruses of the avian leukosis group with an electron dense central nucleoid an intermediate translucent zone and a distinct capsid Very rarely the capsid was composed of two closely approximated membranes separated by a narrow non stained zone Budding virus particles sometimes demon

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strated a translucent centre and no intermediate zone (Fig 8) No particles with deviating morphology were found nor any particles with a significantly larger size or with a doughnut appearance

As a rule there were several particles in the same cell often clustered inside one or a few rather centrally located vesicles in the cytoplasm Virus particles were seen most frequently in cells demonstrating severe damage Thus particles were especially numerous in the central most necrotic parts of the tumours but they could also be demonstrated in other parts of the sarcomas

Virus particles were also seen in the serially transferred RHa sarcomas Fairly rich virus production was observed both in passage no 13 from the new induced tumour line and in passages no 62 95 114 116 124 and 136 from the tumour line propagated at the institute for over 5 years In the third tumour line only 6 transfers have been examined In all of them virus particles were observed although the amount of particles was low and comparable to that seen in the primary tumours Subsequently the production of virus particles was most prominent in the late passages of the serially transferred sarcomas as is exemplified in Figs 3–6 Distinct budding of virus particles was frequently found (Fig 7)

So far no virus particles have been seen in any cell from the serially transferred RHa sarcoma unless it has been severely damaged as seen in the electron microscope (Figs 3–5) No virus particles were seen in primary tumours younger than 14 days and in passage tumours younger than 5 days No virus particles were found in tissue specimens from the injection site before the development of sarcomas

It can also be stated that in the *in vivo* RHa sarcomas the virus production takes place only in the larger cell type described above

The virus particles in the *in vitro* established RHa sarcoma cell showed a somewhat different picture Cell pellets from tumour no 122 and tumour no 139 which were observed ultrastructurally during subculture no 1 2 5 and 7 and 1 5 8 and 11 respectively contained almost invariably several virus producing cells As before virus particles were seen in intra cytoplasmic vesicles in the central fibrillar matrix especially numerous in the border zone between the central part and the rest of the cytoplasm (Fig 9) The virus particle was

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Figs 1–2

Fig 1 Sarcoma cell of smaller type from primary RHa sarcoma This cell demonstrates a proportionally large nucleus with heavy condensation of chromatin along the nuclear membranes The cytoplasm forms irregular and slender processes intermingled with neighbouring cells × 21 600

Fig 2 Sarcoma cell of the larger type from primary tumour Several large mitochondria are seen and the endoplasmic reticulum is abundant Two Golgi systems are also seen The nucleus demonstrates a finely granular chromatin evenly dispersed and only slight condensation is found along the nuclear membranes × 14 000


1 μ
3

sometimes found to be single and sometimes clustered particles were observed in one or several vesicles in the same cell In many vesicles there were chaplet like chains of virus particles budding from the same part of the vesicle membrane apparently not being able to separate from each other (Figs 10 13) As many as 10–15 particles in the same chain could be found Budding of single particles was also found in many cells similar to that seen in the *in vivo* sarcoma Most of the mature and complete particles which had been detached from each other and from the vesicle membrane showed the morphology described above (Fig 10) However several exceptions were observed Virus particles about the same size but with a translucent centre surrounded by a thin membrane like nucleoid were seen and in many particles the circular form was not completed giving the nucleoid the appearance of a horse shoe (Figs 11–12) Some cells showed much larger virus particles (up to 130–140 mμ in diameter) also several twin forms were noted Sometimes small tiny spikes radiating from the dense nucleoid were seen (Fig 10) a finding which was suggestive in some of the particles from the *in vivo* sarcomas as well (Fig 5)

All types of virus particles were seen in the same cell and even in the same intracytoplasmic vesicle In no cells so far examined have virus particles been seen to bud from the outer cell membranes and no particles have been observed extracellularily This was also true in the *in vivo* RHa sarcoma cells

In contrast to the *in vivo* findings virus production *in vitro* was seen in cells that were only slightly damaged The virus producing cells had a cytoplasm which was considerably less electron dense than in the other sarcoma cells and contained a lot of dilated vesicles with ribosomes attached to the outside of the membranes (Fig 9) The central fibrillar matrix was abundant and many of the mitochondria were dilated Several odd shaped forms were also found resembling those described by *Bernhard et al* (1965) and *Bernhard & Tournier* (1966) in adenovirus induced tumours in hamsters The amount of abnormal mitochondria in the virus producing cells far exceeded that seen in the rest of the tumour cells

Biological Tests

Viable RHa sarcoma cells from *in vivo* tumours were injected into the wing web or the pectoral muscle of ten chickens In all of them typical and virus producing Rous chicken sarcomas appeared after a latency period of 14–25 days

---

Fig 3

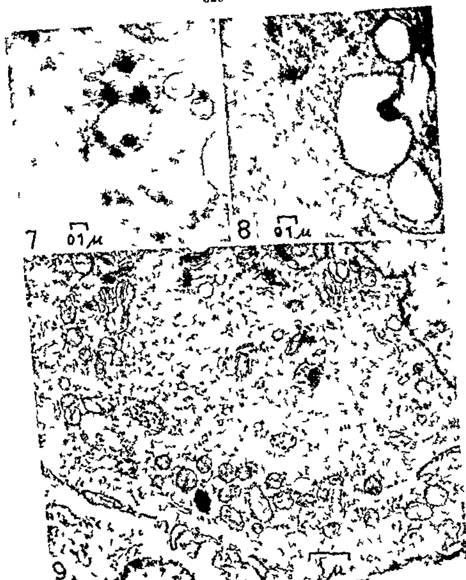
Rous hamster sarcoma cell from serial transfer no 95 Clusters of virus particles in intracytoplasmic vesicles are seen. The cell is totally lacking the outer cell membrane and shows other evident signs of disintegration X 17 000



Figs 4–6

Fig 4 One of the intracytoplasmic vesicles seen in Fig 3 Particle size in this vesicle averages 75 mμ × 58 000

Figs 5–6 RHa sarcoma cell showing clustered virus particles in an amorphous substance inside a partially ruptured intracytoplasmic vesicle Tumour specimen was obtained from passage no 13 in one of the new induced sarcoma lines × 49 500



Figs 7-9

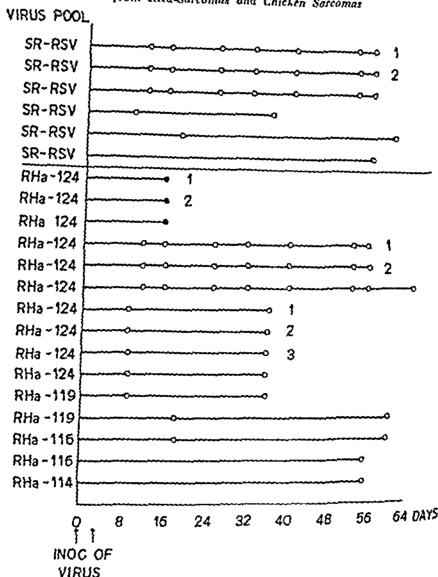
Fig 7 Small intracytopla mic vesicle from RHa passage no 116 *demonstrating* one bu lding virus particle and two adjacent parts of the membrane where the budding process is probably in its initial phase × 49 500

Fig 8 Primary Rous hamster sarcoma cell Budding of a virus particle into an intracyt plasmic vesicle *is seen* *This particle shows* a translucent centre and no distinct intermediate zone × 49 500

Fig 9 RHa sarcoma cell from tumour no 1?? grown *in tissue cultur* This cell is produ ing virus particles and the cytoplasm is characteristically less elec tron-dense than in other RHa sarcoma *cells Th centre* of the cell contains a loose fibrillar matrix Most of the normal cell organelles are distributed at the peripheral margin of this zone *In addition several dilated* vesicles ar found some contain clustere l virus particles × 11 000

TABLE 1

Electron Microscopy of Normal Hamster Fibroblasts Exposed to Virus Suspensions from RHa-Sarcomas and Chicken Sarcomas



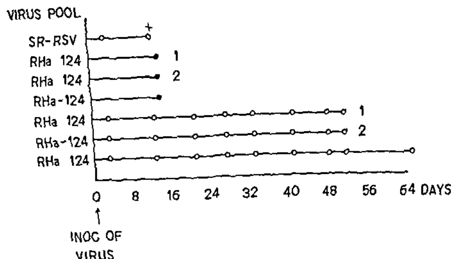
○ Electron microscopy of cell samples
● Electron microscopy of cell samples grown at +33 C.
Sodium hydrocortisone added in conc of 1 10 μg ml of culture fluid 2 1 0 μg/ml of culture fluid. 3 0 1 μg/ml of culture fluid ↓ Virus particles found.

DISCUSSION

The reports on the occurrence of virus particles in the RSV induced mammalian sarcoma cell are contradictory. In most of the original contributions it was stated that the mammalian sarcoma cell retained the RSV genome but when cell free preparations of the sarcoma tissues

TABLE 2

Electron Microscopy of Normal Chicken Fibroblasts Exposed to Virus Suspensions from RHa Sarcomas and Chicken Sarcomas



○ Electron microscopy of cell samples
● Electron microscopy of cell samples grown at +33 C
Sodium hydrocortisone added in conc of 1 10 µg/ml of culture fluid 2 1 0 µg/ml of culture fluid 3 0 1 µg/ml of culture fluid + Virus particles found

were analyzed no infectious Rous virus could be found In the Syrian hamster this was demonstrated *in vivo* (*Ahlstrom & Forsby* 1962 *Shevlyaghin* 1963 1964) and *in vitro* similar results were obtained (*Vesely & Svoboda* 1965 *Vesely et al* 1966) Examination of a RSV induced hamster glioma in the electron microscope did not reveal any virus particles (*Bucciarelli et al* 1967 b) nor were particles found in hamster sarcoma cells cultivated *in vitro* (*Valentine & Bader* 1968)

On the other hand there are some reports that the mammalian Rous sarcoma cell might be able to synthetisize virus *In the* Syrian hamster sarcoma cell this was thought occasionally to occur in primary tumours (*Klement & Vesely* 1963) and also in the serially transferred tumour (*Svoboda & Klement* 1963) Virus production was also observed in rat tumours induced by a chicken sarcoma virus probably related to Rous virus (*Altaner & Svec* 1966) In the electron microscope virus particles morphologically similar to the avian leukosis group have been observed in the Rous meningeal tumour in the dog (*Bucciarelli et al* 1967 a) and in the rat (*Lindberg* 1968 *Valentine & Bader* 1968) No biological tests however have confirmed that the ultrastructurally observed particles are infectious Rous virus

In the present investigation virus synthesis was seen in Rous hamster sarcoma cells *in vivo* and *in vitro* The existence of budding clearly indicates that the sarcoma cells actively produce the virus The par

ticles in some respects are morphologically similar to Rous virus but when the sarcoma cells were grown *in vitro*, several morphological variants were seen which differ from the Rous virus (cf *Epstein* 1956 1958 *Haguenau et al* 1960 1962 *Haguenau & Beard* 1962) In addition the budding process seen *in vitro* did not conform with previous observations on Rous virus elaboration from chicken sarcoma cells (*Dourmashkin et al* 1962 *Heine et al* 1962) The virus particles were seen in almost every sarcoma although in varying amounts Usually the amount of virus particles increased with number of serial transfers The failure to observe particles in some of the primary tumours and early passages was probably due to the fact that the sarcoma specimens were *removed too early in the tumour growth* This was done in order to secure a successful serial transfer which is best achieved when the grafted specimens are as vital as possible This might explain the failure of *Bucciarelli et al* (1967 b) to find any virus particles in the RSV induced hamster glioma the animal may succumb before the tumour has reached the degree of maturity which seems to be necessary for virus elaboration

So far all attempts to demonstrate biological activity of the observed virus particles have proved *completely negative* This was equally the case in the present investigation If it is assumed that the nucleoid of the particles contains the RSV genome the viral capsid produced by the RHa sarcoma cell must have rendered the particle *highly non* infectious This seems less probable as a virus with a capsid produced by a hamster cell ought to be infectious towards other hamster cells which could not be demonstrated

The budding process indicates that the host cells supply at least a part of the viral capsid The viral particle might then acquire new coat antigens and perhaps other biochemical properties which *could* explain the loss of infectivity towards normal chicken fibroblasts (consistent with results obtained by *Hanafusa & Hanafusa* 1966 *Steck & Rubin* 1966 a b *Courington & Vogt* 1967 *Ishizaki & Vogt* 1966 *Vogt & Ishizaki* 1966) For the same reasons however the virus particle might acquire such physical properties that the preparation *and purification methods used in this work are inadequate*

It has been demonstrated that various stocks of Rous virus contain several strains of closely related variants Some of these induce a resistance in the host cell towards superinfections (*RIF*) while other *strains* (*RAV*) *show helper activities* (*Hanafusa & Hanafusa* 1966 *Vogt* 1967) Recently a new strain of RSV demonstrating a high degree of biological non activity has been isolated (*RSV 0*) (*Vogt* 1967) It might be suggested in parallel *to this that during* the infectious cycle in the hamster cell there is a selection of one strain which then is brought to complete maturation

The existence of a latent or an indigenous virus *has been* proved in BHK hamster cells and in hamster cells transformed into malignant

cells by various viruses (adeno polyoma and SV 40 virus) (*Bernhard & Tournier* 1964) The morphology of this virus was found to be similar to the avian leukosis group of virus in some respects but several discrepancies have been noted (*Thomas et al* 1967 *Thomas et al* 1968) So far no reports concerning the biological properties of this virus have been published and at present it is thought to be an indigenous virus variably present in different hamster tumour cells It is suggested that this virus only matures in cells exposed to various external stimuli

A comparative morphological analysis between the virus in the necrotic RHa sarcoma cell and this latent hamster virus is difficult due to technical obstacles in preparing and fixing the necrotic cell material However when studied *in vitro* the RHa sarcoma virus demonstrates several morphological details which coincide with descriptions of the latent hamster virus Thus the variation in size the hollow nucleoid the radiating spikes emerging from the nucleoid and the budding in chains was found to be typical of the "latent virus It is also interesting that when the RHa sarcoma cell is grown *in vitro*, a different morphologic variant of the budding process regularly appeared and also several other characteristics of the latent virus were noted in a higher frequence than *in vivo* This might indicate that some regulatory mechanisms might suppress the virus synthesis *in vivo* i e immunological phenomena

The production of complement fixing antibodies in the *Syrian hamster* carrying a SR RSV induced sarcoma favours the hypothesis that the observed particles are associated with the avian leukosis group of virus It seems likely that the antibody titre will be enhanced if the tumour actively synthetisizes virus particles containing antigens belonging to the group of antigens common to several strains of leukosis virus (gs antigens) As it has been found that the capsid can be interchangeable between related virus strains it might be suggested that the observed particles are coated with a capsid containing antigens from the Rous virus particle but the nucleoid could eventually be derived from an indigenous virus

Thus several morphological findings in vitro and the absence of demonstrable biological activity favour the hypothesis that the RHa sarcoma cells harbour an indigenous virus probably related to or even identical with the virus which has been described by *Bernhard & Tournier* (1964)

Synthesis of a latent hamster virus in the RHa sarcoma cell in contrast to the negative finding in the Rous rat sarcoma cells (*Lindberg* 1968) supports previous opinions that the Rous mammalian cell does not synthetisize infectious Rous virus The contradictory results obtained from different laboratories might be due to the fact that contaminations or indigenous virus strains can be unevenly distributed among the species and among different laboratories

The finding of virus synthesis *in vitro* in morphologically distinct

cells is noteworthy It may reflect that the virus infection converts the cell in a particular way or it might be suggested that this cell is harbouring an indigenous virus infection which is suppressed in the healthy animal When a progressive tumour growth leads to cell necrosis or if the cell is explanted *in vitro* then the virus synthesis may be released The fact that no virus particles were seen in normal hamster cells exposed to the virus but several cells ultrastructurally showed the morphology of virus producers might indicate that the normal hamster fibroblast lacks a promoting factor for the maturation of the virus This unknown factor obviously could be of divergent nature *e g* superinfections with various virus strains both carcinogenic and non carcinogenic Further experiments are needed to clarify the nature of the observed virus In addition the biological role played by the virus and its relation to the Rous virus is open for further investigations

SUMMARY

Three lines of Rous virus induced sarcomas in the Syrian hamster were found to contain virus particles when examined in the electron microscope In addition sarcoma cells explanted and grown *in vitro* regularly synthetisized virus particles The morphology of the virus particle varied but in some respects was similar to the avian leukosis group of virus but especially *in vitro* the virus demonstrated an appearance akin to the latent hamster virus described in several other hamster tumour cells

Experiments were designed in an attempt to demonstrate a biological activity of the observed particles So far no ability to induce sarcomas in chickens or new born hamsters could be detected nor did the virus seem to be infectious towards normal chicken or hamster cells in tissue cultures or *in vivo* It can be suggested from the observations in the electron microscope that the virus particle does not mature in the normal cell but might be able to do so under the influence of various external stimuli applied to the host cell

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COMPARATIVE ELECTRON MICROSCOPIC STUDY OF ROUS SARCOMA IN SYRIAN HAMSTER AND RAT

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Certain strains of Rous sarcoma virus (RSV) are able to induce sarcomas in a wide range of animals The type of tumours varies to a certain extent with the species of animal but two types of cells can usually be distinguished both in the Rous chicken sarcoma and in many Rous mammalian sarcomas a spindle shaped fibroblast like cell and a rounded macrophage like type (*Svet Moldavsky* 1961 *Ahlstrom & Forsby* 1962 *Ahlström & Jonsson* 1962 *Landa et al* 1962 *Klement et al* 1963 *Klement & Svoboda* 1963 *Svoboda & Klement* 1963 *Shevlyaghin* 1963 1964—for comprehensive reviews see *Ahlstrom* 1964 *Zilber* 1965) It might hypothetically be presumed that the cell genome determines certain characteristics of the sarcoma cells while others might be derived from the RSV genome which is known to be present both in the chicken sarcoma and most of the mammalian sarcomas

The ultrastructure of Rous mammalian sarcomas has been described only briefly and mostly in connection with investigations undertaken for special purposes This does not allow a comparison between Rous sarcomas in different mammalian species

The present analysis was carried out in order to elucidate the electron microscopical structure of the two types of cells in the Rous sarcomas of hamsters and rats respectively and in addition the hypothetical presence of common ultrastructural details irrespective of the host

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MATERIAL AND METHODS

Rous sarcoma virus The Rous virus strain Schmidt Ruppin (SR–RSV) has been obtained from chicken sarcomas which were homogenized and purified through ultracentrifugation according to *Moloney* (1960) The virus suspension has been kept at —70 C and the titre of the suspension after storage was 4 4 × 10^5 PFU/ml when tested on chorion allantoic membranes of embryonated leukosis free chicken eggs

Rous sarcomas in the Syrian hamster (RHa sarcoma) Newborn Syrian hamsters were injected with 0 1 ml of the RSV suspension in the lower part of the back Solid partly hemorrhagic sometimes multiple tumours developed at the site of injection in all animals after about 14 days The animals were sacrificed through whole body perfusion technique as described below when the tumours had reached the size of a small pea

Rous sarcomas in the rat (RR sarcoma) New born rats were injected with the same amount and the same stock of Rous virus as used above Tumours appeared after about 3–4 weeks in about 90 per cent of the rats and a few hemorrhagic cysts were also seen When the tumour had reached the size of a pea the animal was killed by whole body perfusion

Fixation through whole body perfusion The animals were anaesthetised by injecting 1 mg of barbital sodium per 100 g of body weight into the peritoneal cavity (Nembutal Abbot Laboratories Ltd Queenborough Kent England) The chest was opened and a small incision was made in the left ventricle of the heart near the apex and a small plastic tube was inserted towards the base of the aorta A ligature was placed around the apex and the perfusion was started as soon as possible After a few seconds the right atrium was opened to allow the perfusate to flow continuously for 20 minutes The hydrostatic pressure of the perfusate was kept between 130–150 cm H_2O The perfusion fluid was saline buffered with Sorensens phosphate buffer and with 2 per cent glutaraldehyde added The final osmolarity of the fixative varied between 300–330 milliosmol (pH = 7 2)

Electron microscopy After the perfusion tumour specimens were excised and cut into small pieces with a razor blade and kept in cold fresh perfusion fluid over night at +4 C The specimens were then washed in 0 1-n cacodylate buffer post fixed in 3 33 per cent OsO_4–s–collidine buffer at +4 C for 60 minutes (*Wood & Luft* 1965) After repeated washings in cacodylate buffer the specimens were dehydrated in graded acetone Most of the specimens were stained during the 70 per cent dehydration step by soaking them in 1 per cent phospho-tungstic acid in ethanol for 15 minutes The specimens were then embedded in Vestopal W according to *Ryter & Kellenberger* (1958)

All sections were cut on a LKB Ultrotome III and mounted on carbon stabilized formvar films covering copper grids Several sections were post stained with lead citrate according to *Venable & Coggeshall* (1965)

The electron microscopic examination has been performed in a Zeiss EM 9 electron microscope equipped with a condensor and all photomicrographs have been processed from Scientia negative film (Gaevert)

Light microscopy Sections about 1 μ in thickness have been processed from all the specimens embedded in Vestopal W They were stained with a solution of 0 1 per cent methylene blue B 0 1 per cent thionin and 0 1 M Na_2HPO_4 in aq dest which was heated to about 80 C in an equal amount of 96 per cent ethanol immediately before the staining The staining time as a rule was 10–30 seconds and the slides were rinsed in distilled water

In addition sections from formalin fixed tumour in a parallel series of animals were processed according to conventional methods

Cytomorphometric methods In order to make a comparative study of the cells seen in the RHa sarcoma and RR sarcoma respectively volumetric calculations have been made from the micrographs based on cytomorphometric principles summarized by *Sitte* (1967) Basic considerations and formulas are also given by *Weibel & Gomez* (1962) *Hennig et al* (1963) *Weibel* (1963) and *Loud et al* (1965)

All determinations have been made by measuring the average cross sectional surface area on the micrographs by means of a transparent sheet of plastic film onto which a lattice of short lines was engraved The pattern of these lines is shown in Fig 1 The plastic sheet was applied to the micrographs and the total length of the lines which transgress the cell the nucleus and the mitochondria was measured



Fig 1

Pattern of lines engraved in a sheet of transparent plastic The film was placed over the micrographs at random and the total length of the lines situated over different cells and cell organelles was determined

For practical purposes all micrographs are considered to be drawn randomly out of a large number of micrographs representing tumour cells ectioned in every possible direction The sampling procedure was thought to exclude the influence of any pre erential direction of the cells in the micrographs although it is obvious from Figs 2 3 and 7 that occasionally the cells are oriented parallel to each other

The degree of homogeneity in the separate group of cells can be estimated from diagrams obtained by plotting the average values of each group of cells and cell organelles against the number of transsected cells With increasing number of transs cted cells the average values approximate towards a straight line It is thought that if cells from an outside population are falsely included in the de terminations this will be dete ted as an irregularity in the diagrams when the average determinations for all parameters in the same group of cells are compared

The surfa e area of the endoplasmic membrane system has been evaluated by determining the number of intersections between the applied lines and the paired membranes in each cell As these are mostly oriented parallel to the long axis of an elongated cell it has been necessary to minimize the influence of this preferen tial direction by rotating the micrographs 60 twice and calculating the average number of three determinations in each cell.

Sampling pr cedure Several pecimens from six hamster sarcomas and approxi mately the same number of specimens from five rat sarcomas were examined in the light microscope Out of these, three specimens from each animal were selected as representative of the tumours and from them 33 micrographs were prepared repre enting the RHa sarcoma and 35 the RR sarcoma The number of cells possible to e aluate in each micrograph varied from 2 to 18 cells The final magnification in all micrographs was 6 100 times

Even in these selected areas several necrotic cells with disrupted cell membranes were found which were not included in the calculations Most of the cells in the different sections have however been measured

As the appearance of the nucleus was the main parameter dividing the cells into different groups in each animal it is obvious that only cells the nuclei of which were transsected were included in the determinations

General consi lerations and volumetric calculat ons The cells in the micrographs are thought to be a random sample of cells from the RHa and RR sarcoma Fur thermore all cell organelles are thought to be randomly distributed in a large total volume of tumour tissue and all components with a special shape (i e the endo plasmic reticulum) are assessed at random by a modification of the evaluation technique

The largest volume which has been determined can be considered small in com parison with the total volume of the tumours

The section thickness is thought to be negligible in comparison with the smallest organelles which are measured

The lengths of the lines across the different cell organelles in the micrographs are directly proportional to the surface area of each component. Thus the average cross sectional surface area for each cell organelle can be calculated and related to the total surface area of the individual type of cells It can be proved that the same relation exists between the volume of the cell organelle and the total volume

The relation between volume V and the average cross sectional surface area S calculated from the micrographs is expressed by the formula $V = \beta \times \bar{S}^{3/2}$ where β is a dimensionless coefficient dependent on the shape of the evaluated body but not on its size The numerical value of β can be calculated when the shape can be approximated by a known geometrical body i e a sphere a cylinder or an ellipsoid (Weibel & Gomez 1962)

If the value of β is not known or cannot be calculated it is obvious from the formula above that in order to compare the volumes of separate cells or cell organelles which have been calculated from their cross sectional surface area in micrographs the values of β for the cells must be close to each other else a systematic error is introduced If it is assumed that the compared cells are reasonably symmetrical the influence of slightly different values of β can be neglected

The morphometric determination is made only on those cells which show a nucleus large enough to allow classification This results in a too large average cross sectional area of the cell in relation to the same area determined from all transsections This overestimation of the average cross sectional surface area can be calculated if it is assumed that the shape of the nucleus located in the centre of the cell is congruent to the shape of the whole cell In Table 1 the corrected figures are given in brackets In addition it can be calculated that if the volumes of two transsected symmetrical cells are compared when the nuclear areas vary from 31 per cent to 44 per cent of the determined average area (values obtained from Table 1) the error in the relation between the volumes is about 8 per cent. It can also be stated that no cell organelles will be over or under represented in the determinations as it is thought that the cell is sectioned in every possible direction and that all cell organelles are evenly distributed

The number of particles per unit volume can be calculated directly from the micrographs if their fractional volume is determined and if the number of transsections of this particular cell organelle per unit area is counted Thus

$$N = \frac{n^{3/2}}{\beta \times p^{1/2}}$$

where N is the number of particles per unit volume (equal size and shape) and n is the number of transsections of these particles per unit area in the micrographs p their fractional volume and β the same dimensionless coefficient as described above depending only on the shape of the particles

In Table 1 the number of mitochondria per unit volume of cytoplasm is given for the different cells assuming that the vast majority of the mitochondria is equal in size and shape and that their shape can be approximated by a sphere The value of β is then 1 382

RESULTS

Light Microscopy

The Rous sarcoma in the Syrian hamster is seen in the light microscope (Fig 2) as a spindle cell sarcoma of varying degrees of maturity often containing giant tumour cells with bizarre nuclei In spite of the pleomorphic cell morphology as a rule one large cell with a vesiculated nucleus and a rich amount of cytoplasm often containing tiny vesicles can be distinguished These cells are intermingled with considerably smaller cells showing hyperchromatic nuclei and small amounts of cytoplasm In the former cell prominent nucleoli can be seen

The Rous sarcoma in rats (Fig 3) appears more homogenous as a



Figs 2–3

Fig 2 Rous hamster sarcoma demonstrating a pleomorphic character with irregular cells somewhat elongated Several large cells with vesiculated nuclei are seen and there are a few cells showing rather chromatin dense nuclei and a small amount of cytoplasm Nucleoli are seen in some cells as well as one cell in mitotic division × 1 000

Fig 3 Rous rat sarcoma seen as a somewhat pleomorphic spindle-cell sarcoma with a few large cells demonstrating chromatin loose nuclei and prominent nucleoli The majority of the cells are smaller showing a small cytoplasmic margin and chromatin-dense nuclei × 1 000

rule, resembling a fibrosarcoma composed of spindle shaped cells arranged in parallel bundles A few giant cells with huge vesiculated nuclei and a rich amount of cytoplasm can be seen In addition the tumour is composed of smaller elongated cells often with chromatin dense nuclei The smaller cell might be irregularly outlined but often has a fibroblast like character

Electron Microscopy

In the electron microscope, it is as a rule possible to confirm the observations made in the light microscope Thus two distinct types of cells are seen in both the RHa and the RR sarcoma It was found that the ultrastructure of the nucleus could be used as a parameter dividing the tumour cells into two main groups They have been designated A cells and C cells respectively while the B cells represent those cells which could not clearly be classified

The Nucleus of Cells Classified as A Cells

This nucleus is generally large rounded or oval with a few infoldings of the rather distinct nuclear membrane often demonstrating several prominent nuclear pores The scanty chromatin is finely granular and evenly dispersed but occasionally small isolated irregular clumps are seen A small electron dense rim of chromatin is seen along the nuclear membrane Usually one or two prominent nucleoli are found in the central part of the nucleus (Figs 4 5 7 8)

The Nucleus of Cells Classified as C Cells

This nucleus is smaller usually slightly oval but sometimes more or less elongated Several deep infoldings are frequently found The nuclear membrane is usually distinct with several and conspicuous nuclear pores The most characteristic feature is the heavy clumping of the chromatin especially along the nuclear membranes appearing as a broad electron dense margin In many cells the aggregated chromatin forms continuous bridges across the nucleus The rest of the chromatin is considerably more electron dense in comparison with that of the A cell nuclei There are nucleoli in some cells but they are never so prominent and distinct as in the nucleus of the A cell (Fig 4 6 7 9)

The Nucleus of Cells Classified as B Cells

Cells with nuclei not distinctly belonging to either group have been classified as B cells (Fig 4)

The Rous Sarcoma in Syrian Hamsters

As seen in Table 1 there were 92 transsected cells classified as typical A cells and 86 as C cells while 25 or approximately 12 per cent



Fig 4

RHa sarcoma Survey of cells of all three type The difference in the appearance of the chromatin is evident, and there is a large amount of endoplasmic reticulum in the A cells The mitochondria appear swollen in this field where several cells are partl disintegrated × 6 900

could not be placed into either group The typical heavy clumping of the chromatin in the C cells can easily be distinguished (Figs 4 6) The nuclear chromatin of the B cells usually resembles that of the A cell nuclei The average cross sectional surface area of the A cell is found to be about 3 times that of the corresponding area of the C cell and their nuclei occupy 32 per cent and 44 per cent of these areas re spectively

The total mitochondrial volume related to the volume of the cyto plasm averages 7 1 per cent and 10 4 per cent in the two types of cells

TABL[E]

Quantitative and Volumetric Evaluations of Cells a[nd]

Tumour cell		No. of transsected cells	Average cell area	Average nuclear area	Nuclear area per cent of average cell area†	"Average cytoplasmic area
	A	92	130 (101)	42	32.2 (42)	88 (60)
RHa	B	25	85	32	38.1	53
	C	86	44 (36)	19	43.6 (53)	25 (17)
	A	43	132 (102)	41	30.9 (40)	91 (62)
RR	B	7	38	18	46.6	20
	C	302	55 (43)	20	36.5 (47)	35 (23)

† In brackets the nuclear volume in per cent of the whole cell volume is given

Using the formula given above and calculating the average number of mitochondria per unit volume in each cell, it is found, however, that approximately the same number of mitochondria per unit volume is seen in both cells: 15.0 and 15.1 respectively.

The morphologic appearance of the mitochondria is rather homogenous and the amount of odd shaped forms is not conspicuous. In both the A and C cells the majority of mitochondria is spherical or slightly oval. In those, which are undamaged and well preserved, a homogenous, occasionally rather electron dense intramitochondrial matrix transversed by a rather few cristae is seen. In some mitochondria a myelin like nest of membranes is found, probably due to inadequate fixation and/or slight cell damage *in vivo*.

The endoplasmic reticulum is more abundant in the cytoplasm of the A cell than in the C cell. When the calculated average figures are converted to number of intersections per unit volume it is found that the endoplasmic reticulum of the A cell is about twice that of the *C cell* (10.9 in relation to 5.2 intersections per unit volume). Generally the endoplasmic reticulum is seen as a "rough" surfaced system of paired membranes and only in the immediate neighbourhood of a Golgi complex can the membranes be smooth. Occasionally the paired membranes are separated forming vesicles containing amorphous substance.

The Rous Sarcoma in Rats

There were only 43 transsected cells classified as typical A cells among 387 cells in total (= 11 per cent). Of the remaining cells only 7 were impossible to accurately classify (= 2 per cent), while the vast majority of cells, 302 out of 352, has been assigned as C cells (the rest have not been determined due to technical reasons). The nuclei of the A cells always have the typical appearance very similar to that of the A cell in the RHa sarcoma. The nucleolus also may look like a mirror

Organelles in Primary Rous Hamster and Rat Sarcoma

Average mitochondr area	Mitochondr area per cent of cytoplasm	Average no of mito chondria per cell	No of mito chondria per unit volume of cytoplasm	Average no of intersected endoplasmic membranes	No of intersections per unit volume of cytoplasm
6 3	7 1	2 76	15 0	9 6	10 9
2 8	–	–	–	3 0	–
2 6	10 4	0 89	15 1	1 3	5 [illegible]
8 4	9 3	2 33	9 7	13 4	14 8
4 7	–	–	–	2 7	–
4 5	1[illegible] 9	1 06	10 6	3 6	10 2

In brackets the corrected average areas are given

image of its counterpart in the A cell of the RHa sarcoma The nucleus of the C cell in the RR sarcoma is not so characteristically chromatin dense as in the RHa sarcoma The chromatin always shows clumping but not to the same extent as in the RHa sarcoma and chromatin bridges across the nucleus are seldom found (Figs 7–9) The morpho logy of the B cells is usually found to be more akin to that of the C cells

The average cross sectional surface area of the A cell of the RR sarcoma is almost exactly as large as in the A cell of the RHa sarcoma while this area in the C cell of the RR sarcoma is about 25 per cent larger than in the hamster The RR nuclei occupy 31 per cent of the average cell area in the A cell and about 37 per cent in the C cell However the nuclei of the C cells and the A cells in the RR sarcoma have almost the same average area as in the corresponding cells of the RHa sarcoma

The mitochondrial volume averages 9 3 per cent of the cytoplasm of the A cell and 12 9 per cent of the C cell The calculated numbers of mitochondria in the A cell and the C cell of the RR sarcoma are also found to be equal 9 7 and 10 6 per unit volume of cytoplasm

The endoplasmic reticulum is more abundant in the A cell in the RR sarcoma (Table 1) The number of intersections per unit volume of cytoplasm in the A cell is about 1 5 times higher than in the C cell The RR sarcoma cells however have larger surface areas of the endo plasmic reticulum per unit volume of cytoplasm in comparison with the corresponding type of cells in the RHa sarcoma

The morphological appearance of the mitochondria and of the endo plasmic reticulum in the two types of RR sarcoma cells do not differ essentially from that in the RHa sarcoma cells The number of swollen or deranged mitochondria is less in the RR sarcoma cells than in the RHa sarcoma cells and consequently no myelin like figures in the mitochondria of the RR sarcoma cells are found The average number


5
1μ
6
1μ

of mitochondrial cristae in the RR cells seems to be slightly higher although no counts have been made. In addition there seems to be slightly more rod shaped or cylindrical mitochondria in the RR sarcoma cells but this might be a false impression as due to osmotic forces damaged mitochondria in the RHa sarcoma cells attend a spherical shape. In the RR sarcoma mitochondria a moderate or rather dense matrix can be seen between the cristae.

The B cells of neither the RHa nor the RR sarcoma show any particular characteristics as regards the mitochondria or the endoplasmic reticulum. The values are not summarized as the low number of cells will make the calculations unreliable especially in the RR sarcoma.

Other Cell Constituents in the RHa and the RR sarcoma

A prominent nucleolus is almost invariably found in the A cell of the RR sarcoma cells but the frequence is not so high in the A cell nuclei of the RHa sarcoma. Most nuclei usually show only one nucleolus but two or even more may be found. The nucleolus of the A cell is characteristically located in the centre of the nucleus and approximation towards the nuclear membranes is rare. The shape of the nucleolus is almost spherical but might be slightly oval in elongated nuclei. A typical nucleolus is seen in Fig 8 from an A cell nucleus of a RR sarcoma cell.

In the C cells the nucleoli are not seen as frequently and as regularly as in the A cells. The number is found to be somewhat higher *in the C* cells of the RR sarcoma. The nucleoli are sometimes multiple *as a rule* not so distinctly outlined as in the A cells and their shape can be rather irregular.

The Nuclear Membrane

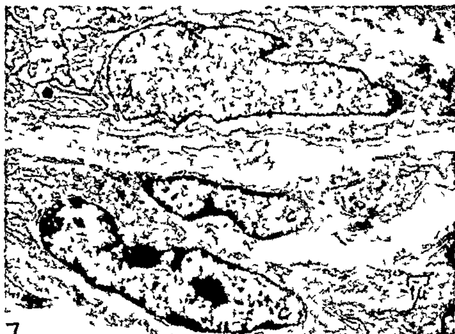
The nuclear membranes are very distinct in all types of cells and are interrupted at regular *intervals* by nuclear pores. No difference in this respect has been found between the A and the *C cells* nor is there any difference between the *species*. The membranes show in both species many and deep infoldings especially in the *C cells*. An irregular space between the two membranes can be seen in some cells. It has been

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Figs 5–6

Fig 5 Sarcoma cell *from* the Rous sarcoma in the Syrian hamster. A typical A cell demonstrating a small amount of marginated chromatin along the nuclear membrane where distln t nu lear pores are seen. In the centre of the nucleus there are *crystalline* like aggregates. In the cytoplasm the rough surfaced" endoplasmic ret culum is conspicuous × 9 100

Fig 6 Irregular RHa sarcoma cell *type C*. The condensed and marginated nuclear chromatin forms coalescent lumps in the nucleus. The cytoplasm forms long slender *processes* containing tiny ve icles × 19 000


c
c
1μ
7
8
1μ

regarded as a sign of necrosis *in vivo*, as inadequate fixation does not regularly create this type of artefact

The Golgi System

The Golgi system is regularly found in most of the A cells in both the RHa and the RR sarcoma It is also seen in some of the C cells in the RR sarcoma but is an exceptional observation in the C cell of the RHa sarcoma In the large RHa sarcoma cells they are usually multiple and are seen as convoluted and lamellar membranes of smooth appearance often associated with numerous small vesicles and occasionally large vacuoles As a rule the Golgi system is found in the peri nuclear part of the cytoplasm and mostly in the centre of the cell

The Lysosomes

Only very few and rather small lysosomes are present in the primary sarcoma cells of both the hamster and the rat They are more common in the C cells in both species The average volume of the lysosomes can roughly be estimated to be under 0 1 per cent of the cytoplasmic volume in cell type A and under 0 5 per cent in cell type C

Free Ribosomes and Fibrillar Cytoplasmic Matrix

Free ribosomes appearing as polyribosomes are seen in all of the tumour cells Judging from the micrographs it might be concluded that there is a larger amount in the A type of cells in both species

The fibrillar matrix described elsewhere (*Bernhard & Tournier* 1964) has been seen in a few of the A type of cells but only in the RHa sarcoma

The Cell Membrane

The sarcoma cells are delimited by a plasma membrane of normal appearance No morphologic details deviating from normal cells have been observed Especially in the C cells long and slender cytoplasmic processes are formed which intermingle with several neighbouring

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Figs 7–8

Fig 7 Survey of Rous rat sarcoma In the upper part a large cell type A is seen, and below two cells belonging to the C group The difference in appearance of the nuclear chromatin is evident × 5 900

Fig 8 Nucleus of typical A cell in the Rous rat sarcoma Only a tiny rim of elec tron dense chromatin is seen along the nuclear membrane The nucleolus is very prominent in the finely granular chromatin A large amount of cyto plasm containing a well-developed endoplasmatic reticulum is seen Several small mitochondria are seen as well as part of a small Golgi system × 17 700

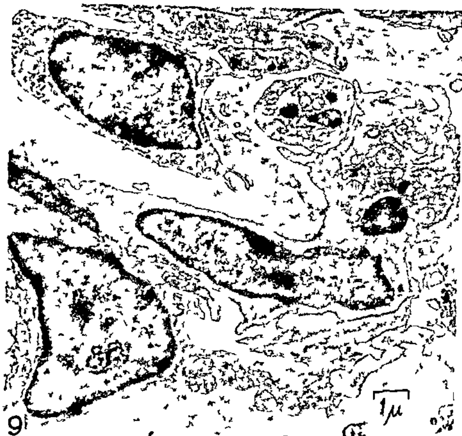


Fig 9
RR sarcoma demonstrating three rather irregular cells of type C The clumping of chromatin as well as the prominent condensation along the nuclear membrane is obvious Only few endoplasmic membranes are found and the mitochondria are rounded containing interdigitated cristae × 9 700

cells In the solid sarcoma no true microvilli are seen in contrast to the *in vitro* findings in explanted sarcoma cells (*Febvre et al* 1964 *Lindberg* 1967)

Other Morphologic Observations

In the A cell type of nuclei in the RR sarcoma but more frequently in the RHa sarcoma various morphologic details of unknown origin are seen Small granular crystalline like aggregates can be observed occasionally surrounded by a membrane like condensation of the nuclear chromatin (Fig 4)

In a few of the A cells mostly in the RR sarcoma condensation of a fibrillar material just inside and parallel to the cell membrane has been observed This phenomenon has been described in normal fibroblasts i e in collagen forming mouse fibroblasts in tissue cultures

(*Goldberg & Green* 1964) and in virus producing chicken fibroblasts (*Courington & Vogt* 1967)

Residual bodies mostly as myelin like figures are infrequently found No obvious difference in this respect exists between the two cell types or between the two animals

No crystalline material has been found in the cytoplasm of any sarcoma cell in either animal

Accuracy of the Volumetric Determinations

The average cross sectional surface area is dependent on both the size and the shape of the transsected cells In order to compare volumetric calculations of different groups of cells either the size or the shape of the cells must be known If it is assumed that within each group of cells the variations in size are minimal a good estimation of the dimensionless coefficient β can be obtained by plotting the determined areas in a distribution diagram and by calculating the relative standard deviation within each group It can be proved that the relative standard deviation is independent of the size of the cells The relative standard deviations of the nuclear areas in the present material are numerically so close that it can be assumed that the values of β are the same Comparing the same values for the "average cell areas it is found that the RR sarcoma C cell might be approximated by a sphere while the other cells have their relative standard deviation comparable to that of the nuclear areas and here the nuclear shape is congruent with that of the cell However the differences are so small that assuming the same value of β for all cells and their nuclei, the error can be neglected in this investigation

TABLE 2

Percentage Change of Average Determinations of Cells and Cell Organelles in Rous Sarcoma of Hamster and Rat with Increasing Numbers of Observations

Cell	Total area	Nuclear area	Mitochondrial area	No of endoplasmic membranes
RHa-A	−7	−4 5	−8	−15
RHa C	−7	−2 5	+8	−9
RR A	+7	−3 5	−7	−5
RR-C	−2	−3	+2	−8

As the appearance of the nucleus was the only disparating parameter of the different cell groups it is necessary to ascertain that no cells from an outside population are included A remarkably low variance was found in each group of cells of all parameters In Table 2 the percentage change of the average calculations are given for all parameters when the number of observations progressed from 80 per cent

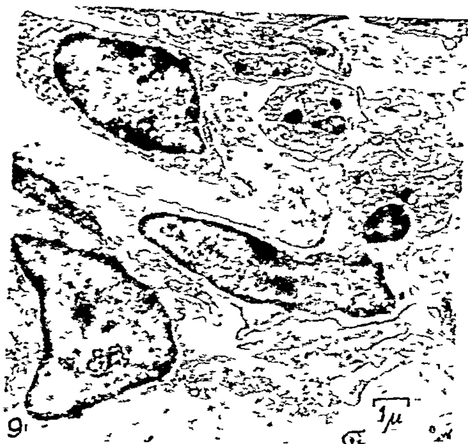


Fig 9

RR sarcoma demonstrating three rather irregular cells o type C. The clumping of chromatin as well as the prominent condensation along the nuclear membrane is obvious. Only few endoplasmic membranes are found and the mitochondria are rounded containing interdigitated cristae. × 9,200

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Percentage Change of Average Determinations of Cells and Cell Organelles in Rous Sarcoma of Hamster and Rat with Increasing Numbers of Observations

Cell	Total* area	Nuclear area	Mitochondrial area	No of endoplasmic membranes
RHa A	−7	−4.5	−8	−7.5
RHa-C	−7	−2.5	+8	+8
RR A	+5	−3.5	−7	−5
RR C	−2	−3	+2	−8

As the appearance of the nucleus was the only disparating parameter of the different cell groups it is necessary to ascertain that no cells from an outside population are included A remarkably low variance was found in each group of cells of all parameters In Table 2 the percentage change of the average calculations are given for all parameters when the number of observations progressed from 80 per cent

to 100 per cent of the whole population in each group Less than 10 per cent change was seen which favours the assumption that the cell population in each group is rather homogenous

DISCUSSION

In most of the ultrastructural studies of both human and experimental tumours, the quantitative evaluation of cells and cell organelles is expressed in terms of a large amount of very rich in or is scanty which is then illustrated with a few and selected micrographs This makes any attempt to compare one tumour with another rather speculative Using cytomorphometric methods more objective information can be obtained from the micrographs

One difficulty in using morphometric methods in the electron microscopical analysis of a malignant tumour is the fact that only a very small fraction of tissue can be studied The sampling procedure might be influenced by many factors difficult to control i e non homogenous perfusion of the fixatives local variations in the osmolarity of the extracellular fluids cell necrosis inadequate vascular supply causing anoxic damage interrupted lymph drainage abnormal pH from predominant anaerobic metabolism and several other factors In the present investigation the sampling procedure is based mainly on evaluation of several tissue blocks in the light microscope from which suitable specimens have been selected for further analysis in the electron microscope It is well known that the Rous mammalian sarcomas show wide variations in their morphology and this is evident especially in the Syrian hamster As far as possible representative areas from hamster and rat sarcomas have been selected for comparison

Previous Reports on the Ultrastructure of Rous Mammalian and Chicken Sarcoma Cells

The ultrastructure of the Rous mammalian sarcomas has been described in only a few investigations

The Rous hamster sarcoma in the brain is reported to be composed of two types of cells (*Bucciarelli et al* 1967 b) and it was concluded that one type probably represented an abnormal astrocyte while the other was not classified The description of the Rous brain sarcoma cells seems to be in keeping with the appearance of the A cell and C cell in the present study The RR sarcoma cell in tissue culture is described as a polygonal irregular fibroblast like cell producing fibrils of collagen (*Febvre et al* 1964) The RR sarcoma *in vivo* was found to be composed of two types of cells one fibroblast like and one smaller electron dense thought to be more undifferentiated (*Lindberg* 1968)

The ultrastructural appearance of a few other mammalian sarcomas

have been briefly described The Rous monkey sarcoma is composed of two cells one rounded and one elongated fibroblast like cell (*Munroe et al* 1964 *Rabotti et al* 1967) In the dog a lepto meningeal sarcoma was described as pleomorphic Pale and dark cells occasionally wrapped around each other were found and intracytoplasmic fibrils were seen in bundles near the plasma membrane (*Bucciarelli et al* 1967 a)

Epstein (1957) gives a detailed ultrastructural analysis of the Rous chicken sarcoma cells A large fusiform fibroblast like cell is found together with varying amounts of rounded macrophage like cells The former contains a nucleus typical of fibroblasts with a finely granular chromatin and well demarcated nuclear membranes The mitochondria were rod shaped relatively few and the amount of cristae and the mito chondrial matrix scanty

Later publications essentially confirm the morphological description (i e *Haguenau & Beard* 1962) In Rous virus producing cells slight or moderate clumping of the chromatin can be found in contrast to the normal cells (*di Stefano & Dougherty* 1965) The number of mitochondria has been described both as increased (*Goldé* 1959 *di Stefano & Dougherty* 1965) as unchanged (*Hampton & Eidinoff* 1962) and as decreased (*Gaylord* 1955 *Bernhard et al* 1956 *Epstein* 1957 *Covington & Vogt* 1967) Most observers agree that several atypical mitochondria are seen The Golgi system is generally found to be hypertrophic and the endoplasmic reticulum is well developed especially in the large cells In virusproducing chicken cells dense bodies are found and structures thought to be related to the production of virus have been described even the existence of pro virus in particle like shapes has been suggested (*Haguenau* 1960 *Haguenau et al* 1960 1961 1962 *Haguenau & Beard* 1962)

It seems that the Rous sarcoma both in chickens and mammals is invariably composed of two types of cells One cell is generally larger and is described as fibroblast like showing some ultrastructural details usually seen in normal fibroblasts This cell is found to have a rather large amount of cytoplasm and the cell demonstrates a sub cellular morphology consistent with high metabolic and/or synthetic activity The other cell is described as a small electron dense cell irregularly out lined but more rounded in appearance Occasionally this cell has been described as macrophage like but there are no ultra structural findings supporting this view

Comparison of the Ultrastructure and the Morphometric Findings in the Rous Hamster and Rous Rat Sarcoma Cell

A volumetric analysis of the Rous sarcoma cells from micrographs can be influenced by many extraneous factors It has been pointed out that the osmolarity of the fixatives is of great importance when whole body perfusion methods are employed for the primary fixation of the

tissues (*Maunsbach* 1966 *Sjostrand* 1967) Of several buffer systems tried in pilot studies none was found to be *superior to a fixative based* on a commonly used buffered saline solution with 2 per cent glutaraldehyde added and with the total osmolarity close to the tonicity of serum It is thought that the total cell volume the nuclear volume and the total surface area of the endoplasmic reticulum has not been artificially altered in the tumours by the fixative although the tonicity might not have been optimal The influence on the mitochondrial volumes however is more difficult to evaluate but in a comparative study this can be partly overcome by converting the determinations of volumes to number per unit volume of cytoplasm using a calculation procedure where the influence of erroneous volumetric determinations is minimized As seen in Table 1 this procedure transforms the slightly disparate volumetric figures into two homogenous groups one for each animal It seems probable that the mitochondrial volume is influenced by artefacts Judging from the micrographs the RHa sarcoma in general shows the greater number of damaged cells It can therefore be expected that an artificial swelling of the mitochondria will be higher in the RHa sarcoma cells than in the RR sarcoma cells Thus the percentage volumes in the two species would in fact differ more from each other In addition it cannot be excluded that a certain number of abnormal mitochondria may have reacted in unpredictable ways towards the fixatives (cf *Bernhard et al* 1965 *Bernhard & Tournier* 1966)

The condensation and aggregation of chromatin in the nucleus is dependent on the fixatives used and is admittedly an arbitrary parameter dividing the cells into different groups In spite of this it must reflect certain physical or biochemical differences between the cells The reactions of the chromatin are reproducible even in malignant cells as demonstrated in the present investigation The morphometric evaluation of the cell organelles demonstrated that the different appearance of the nuclear chromatin rather strictly corresponds to a specific organisation and morphology of the cell organelles indicating that the artificial reactions reflect different biological properties of the cell

Specific cytomorphologic patterns common to the Rous sarcoma cells were found in both the hamster and the rat Apart from the similarity in the nuclear morphology the A cells in both the RHa and the RR sarcoma have about the same volume and the same nuclear volume In addition the mitochondrial volumes in both cells are in the same range The well developed endoplasmic reticulum found in the A cells of both species is also a conspicuous finding In addition the C cells of both species are similar in many respects The nuclei are about equal in volume and thus the difference in cell volumes is due to a larger cytoplasmic volume of the RR sarcoma C cell It is noteworthy that in this cell the difference is paralleled by an absolute increase of the total

surface area of the endoplasmic reticulum per unit volume of cytoplasm in comparison with the C cell in the RHa sarcoma The higher frequence of nucleoli in the RR C cell is also consistent with the assumption that the C cell of the RR sarcoma is more active in its metabolic and/or synthetic functions The mitochondrial volumes in the two C cells are also in the same range

Another pattern which transgresses the host barrier is the morphology and character of the A cell in relation to the C cell In both the RHa and the RR sarcoma the A cell demonstrates ultrastructural traits indicating high synthetic and/or metabolic activity while the C cells demonstrate a morphology suggestive of a much lower degree of metabolic turnover possibly a resting state The A cell compared with the C cell shows in both species about a 3 fold larger volume of the nuclei about 3 5–5 times larger volume and between 1 5–2 times larger surface area of the endoplasmic reticulum and this is parallelled by the appearance of prominent nucleoli and hypertrophic Golgi systems

The number of mitochondria has been found to vary only slightly between tumour cells in the same animal It is about 15 per unit volume of cytoplasm in the hamster sarcoma cell and about 10 in the rat sarcoma cell As the mitochondria perform the aerobic metabolism of the cell thus producing the energy supply it might be expected that the metabolic turnover is greater in tumours in the Syrian hamster than in the rat This is in good agreement with the much faster growth of the tumour generally noted in the hamster The lesser number of mitochondria in the RR sarcoma cell however is at least partly counterbalanced by their larger volume

As is seen in Table 1 the B cells are too few in the RR sarcomas to allow any conclusions to be drawn from the volumetric determinations In the RHa sarcoma these undecided cells are more numerous and in many respects are transitional cells They are intermediate between the A cell and C cell not only as regards the size of the cells but also as regards the size of their nuclei and the amount of the endoplasmic reticulum

The biological properties of the two types of cells are difficult to evaluate from the results of the present morphometric and morphologic investigation It has been suggested that both cells are sarcoma cells as they seem to be represented in the same proportions in distant metastasis in various organs as in the primary tumours (Ahlstrom & Jonsson 1962) It seems likely that the A cell is a true sarcoma cell with malignant properties although the cell in many respects is similar to a normal fibroblast The C cells show a varying morphology suggestive of malignancy at least as seen in the electron microscope In addition the high percentage of this cell type encountered in the RR sarcoma favours the hypothesis that the C cell is integrated with the tumour growth and not a proliferating normal fibroblast responding to the invasive growth of the sarcoma tissue

It cannot be excluded that a few normal cells could have been included in the volumetric determinations as it has been shown that stroma cells rapidly integrate in an invasive growth of Rous tumours (*Mark* 1967). As the analysis of the accuracy of the determinations shows however it seems liable that the amount is low.

If it is assumed that both cells are tumour cells the volumetric characterisation might indicate that the C cell could be a metabolic less active variant of the larger A cell. The tumour might produce its own stroma by modifying a varying amount of the sarcoma cells from metabolic active proliferating cells into still malignant but less active cells. This is accomplished by reducing the cytoplasmic volume, the amount of endoplasmic reticulum, the nuclear volume, the nucleolus and the hypertrophic Golgi zone, while other constituents of the cell, probably more basic, are retained, i.e. the general shape of the cell and the nucleus, the amount of nuclear pores, the morphology and number of mitochondria. This hypothesis has also been suggested from *in vitro* studies where it was thought that the two types of cells were interchangeable (*Doljanski & Tenenbaum* 1941).

SUMMARY

Primary sarcomas in the Syrian hamsters and rat induced by Rous sarcoma virus (strain Schmidt-Ruppin) were studied in the electron microscope. It was found that in both the hamster and in the rat the tumours were composed of two types of cells with characteristic ultrastructural morphology. Using cytomorphometric methods and calculating the volumes for different cell organelles and cells it was found that in both the hamster and the rat there are specific cytomorphologic patterns which resemble each other over the species barriers.

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INTRARENAL VASCULAR ALTERATIONS AND THE PERSISTENCE OF EXPERIMENTAL HYPERTENSION

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It is a common experience that hypertension due to unilateral renal artery stenosis is less likely to respond favourably to surgical correction the longer it has been present This feature is also well documented in the experimental animal On the basis of their classical experiments *Wilson & Byrom* (1941) concluded that the blood pressure elevation would in the end induce arteriolosclerotic changes in the contralateral kidney which would be capable of maintaining a form of renal hypertension even after removal of the kidney with renal artery stenosis This hypothesis has later gained support by experimental results obtained by *Floyer* (1951) and observations in human beings (*Vertes et al* 1965) *Fisher et al* (1968) point out however that although intrarenal arteriolosclerosis may promote the development of a renal phase of hypertension it can hardly be the primary defect in hypertensive disease since it appears also in a number of normotensive disorders Others maintain that neither histological features nor functional data support the view that arteriolosclerosis in the contralateral kidney is of importance for the persistence of the hypertensive state (*Flasher et al* 1951)

Previous studies on kidneys from human beings with essential hypertension have shown that the degenerative glomerular lesions which occur in these kidneys will induce specific alterations in the intrarenal micro angioarchitecture (*Ljungqvist* 1962) These alterations are unrelated to arteriolosclerosis and may form a morphological basis for the development of a renal phase of hypertension The present experiments were undertaken to see whether similar alterations would develop in the vasculature of the contralateral kidney of rats with hypertension due to unilateral renal artery stenosis For this purpose rats were made hypertensive by unilateral renal artery stenosis and stereomicro angiographic examinations performed on the contralateral kidney both from rats in which removal of the kidney with renal

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artery stenosis did not lower the blood pressure and from rats in which the nephrectomy resulted in normalization of the blood pressure Since it is known that the degree of granulation of the juxtaglomerular cells in the kidney undergoes characteristic changes in various forms of hypertension (*Tobian* 1966) the study included a determination of the degree of juxtaglomerular granulation in the kidneys

MATERIAL AND METHODS

Fifty female Sprague Dawley rats were used They were kept on tap water *ad libitum* and a commercial diet containing 0 4 per c nt sodium chloride

At the beginning of the experiments the rats weighed between 180 g and 200 g and their blood pressures were normal This was assessed on three different occasions using the tail pletysmographic method (*William et al* 1939) and with the rats under brief ether anaesthesia The rats were divided into two groups and subjected to operation

Group I In these 40 rats a left lumbar incision was made und r ether anaesthesia and a silver clip placed around the left renal artery close to its origin from the aorta The internal gap of the clip was 0 15 mm The blood pressures were then measured at weekly intervals Hypertension was considered to be present when the blood pressure became stabilized at or above 140 mm Hg provided that this included a rise by at least 15 per cent of the initial blood pressure level

In 29 rats hypertension developed within 7 weeks following operation After hypertensive periods of 2–39 weeks the clipped kidney was removed followed by weekly control of the blood pressure for a further 8 weeks According to the effect of nephrectomy on the blood pressure the rats were divided into two subgroups *subgroup A* consisted of 15 rats which remained hypertensive after removal of the clipped kidney and *subgroup B* of 14 rats in which the blood pressure dropped to normal value

In eight rats clipping of the left renal artery did not result in any blood pressure alterations These rats were subjected to left nephrectomy 10 weeks after renal artery clipping and followed for a further 8 weeks They constitute *subgroup C* of the Group I rats

In three rats the blood pressure fluctuated between hypertensive and normotensive levels following renal artery clipping These rats were excluded from the material

Group II In this group of 10 rats a sham operation was performed including a left lumbar incision under ether anaesthesia and the application and immediate removal of a 0 15 mm wide silver clip on the left renal artery Ten weeks after the operation the left kidney was removed and after another 8 weeks the rats were sacrificed The blood pressures were measured once a week

When the left kidney of the rats of Group I was exposed for removal it was ascertained by careful inspection that the clip was still in correct position After removal of the left kidney this was injected with a 10 per cent aqueous suspension of fine grain barium sulphate (Micropaque) and processed for a combined stereo micro angiographic and histological examination as previously described (*Ljungqvist* 1963) The findings in these kidneys will be the subject of a later report In the present study however a determination of the degree of juxtaglomerular granulation of the left kidneys was included to see whether this would differ from that of the corresponding right kidneys For this purpose the kidneys were fixed in a modified Helly's solution (*Ljungqvist & Richardson* 1963) after the injection of contrast medium Thin sections of these kidneys were then stained with the Bowie method for juxtaglomerular granules The degree of granulation was determined by calculating the juxtaglomerular index (JGI) according to *Hartroft & Hartroft* (1953)

The rats were killed by the injection of a 10 per cent aqueous suspension of Micropaque into the arterial system during ether anaesthesia (*Ljungqvist* 1966) The right (untouched) kidneys were then processed for combined stereomicro angiographic and histological examinations including staining of histological sections with the Bowie method for juxtaglomerular cell granules

Since it was found that glomerular lesions developed in the untouched kidney in the rats of Group I (see results) and there appeared to be differences between the various subgroups in the degree and extent of these lesions a glomerular lesion index (GLI) was determined for each of these kidneys This included the counting of all glomeruli in a frontal section of the kidney and evaluation of the degree and extent of the lesion in each glomerulus affected The glomerular lesions were either focal or the entire tuft was affected to a varying degree. A glomerulus was considered slightly involved (+) when alterations regardless of severity were restricted to 1/3 of the tuft and less or when the entire tuft was slightly affected (Fig 1) Moderate glomerular changes (++) indicate that 1/3 to 2/3 of the tuft was affected, or that the entire tuft was moderately altered (Fig 2) Severe changes (+++) indicate that more than 2/3 of the tuft was severely affected The number of glomeruli denoted as (+), (++) and (+++) was multiplied by factors 1 2 and 3 respectively The sum of these products per 100 glomeruli constitutes the GLI

RESULTS

Clinical Findings (Table 1)

In no rat was the blood pressure at start of the experiments above 135 mm Hg All groups of rats gained weight steadily during the experiments This gain was slower in the presence of *hypertension* the increase in weight in group I A and I B was recorded over average periods of 38 and 29 weeks and that in groups I C and II over a period of 18 weeks

In the rats in group I A hypertension had been present for an average period of 23 weeks when left nephrectomy was performed At this time the blood pressure range for the rats was 145–210 *mm Hg (mean* 180 mm Hg) When the animals were killed the range was 140 225 mm Hg (mean 170 mm Hg)

TABLE 1

Clinical Data of rats with Left Renal Artery Stenosis and Persistent Hypertension (I A) Reversible Hypertension (I B) and Normotension (I C) A Control Group of Sham Operated Rats is Included (II) The Figures are Mean Values

Group of rats		No of rats	B p¹	B p²	B p³	Weight¹	Weight³
I	A	15	110	180	170	190	295
	B	14	110	160	115	195	310
	C	8	115	110	115	195	315
II		10	115	110	115	180	290

B p¹ Blood pressure at start of experiment
B p² Blood pressure at left nephrectomy
B p³ Blood pressure at end of experiment
Weight¹ Body weight at start of experiment
Weight³ Body weight at end of experiment

In the rats in group I B hypertension had been present for an average period of 14 weeks when left nephrectomy was performed At this time the blood pressure range of the rats was 140–185 mm Hg (mean 160 mm Hg) When the rats were killed the range was 90–135 mm Hg (mean 115 mm Hg)



Figs 1–2

Fig 1 Cortical glomerulus from the right kidney of a rat in which hypertension persisted after removal of the clipped left kidney (Group I A) There is (+) glomerular lesion with occlusive capillary alterations in top right part of the tuft The unaffected glomerular capillaries contain contrast medium Note that the contrast filled afferent arteriole (larger arrow) and the empty efferent arteriole (smaller arrow) are distinctly separate vessels Cf Figs 6 B and 10 Ladewig × 450

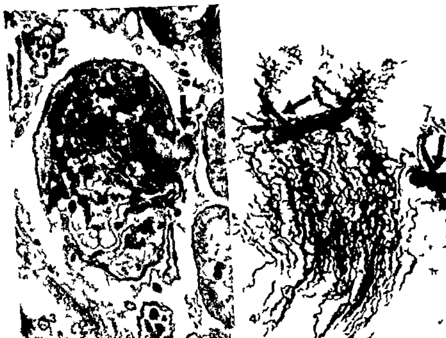
Fig 2 Cortical glomerulus from the right kidney of a rat in which hypertension persisted after removal of the clipped left kidney (Group I A) There is (++) glomerular lesion with occlusive capillary alterations in top half of the tuft The unaffected part of the tuft and the afferent arteriole (right) contain contrast medium Cf Fig 10 Ladewig × 450

In the rats in groups I C and II no significant blood pressure alterations were recorded during the experimental period

Morphological Findings (Table 2)

Persistent hypertension (Group I A) In the untouched kidney in these 15 rats the preglomerular arteries and the medullary vessels were usually well visualized in the micro angiograms whereas the filling of the glomeruli and the postglomerular cortical vasculature was incomplete (Fig 4) Veins were clearly visualized in the juxta medullary zone and inner cortex but rarely in the more peripheral parts of the cortex (Figs 4 5A and 7A)

In the *cortex* the micro angiographic pattern of many of the visualized glomeruli was simplified to a varying degree and their post



Figs 3 4

Fig 3 Cortical glomerulus from the right kidney of a rat in which hypertension persisted after removal of the clipped left kidney (Group 1 A) There is (+ + +) glomerular lesion with occlusive capillary alteration in the entire tuft This contains practically no contrast medium whereas the afferent arteriole (arrow) is filled. Cf Fig 10 Ladewig × 450

Fig 4 Micro angiogram from the right kidney of a rat in which hypertension persisted after removal of the clipped left kidney (Group 1 A) The arteries (larger arrows) and veins (smaller arrows) in the juxtamedullary zone are well visualized In the cortex the arterioles end blindly and there is hardly any visualization of glomeruli and peritubular capillaries The vasculature of th medulla is completely visualized Cf Fig 8 × 30

glomerular vessels were not seen (Fig 5 A) Other glomeruli were not visualized at all their afferent arterioles ending blindly (Fig 5 A) In the histological sections it was found that the simplified and non visualized cortical glomeruli were partially or completely degenerated (Figs 1 3 and 5 B)

In the *juxtamedullary zone* numerous arterioles were seen in the micro angiograms to have a simplified glomerular tuft (Fig 6 A) or to be entirely devoid of a tuft (Fig 7 A) Nevertheless the postglomerular parts of these arterioles were well visualized and were seen to split up into arteriolae rectae leading to the medulla In the histological sections the micro angiographic features of the juxtamedullary glomeruli were found to be due to partial (Fig 6 B) or complete degeneration of the tufts (Fig 7 B)

Both in the cortical and juxtamedullary zones the glomerular de



Fig 5

a Micro angiogram from the inner cortex of the right kidney of a rat in which hypertension persisted after removal of clipped left kidney (Group I A) One glomerulus appears normal (bottom right) but most glomeruli show various degrees of simplification of their capillary patterns (larger arrows) Numerous arterioles end blindly Venous filling is prominent (smaller arrows) × 75

b Histological section of the glomerulus at middle arrow in Fig 5 a showing occlusive alterations of the tuft This is only partially filled with contrast medium Capsule adhesions are seen Top right there is proliferation of the capsule with involvement of part of the tuft Ladewig × 450

generation consisted in hyaline or fibrinoid thickening of the capillary walls leading to occlusion of the lumina and fibrosis and hyalinization of the tuft These lesions were often focal with partial or even complete occlusion of the capillaries in part of the tuft (Figs 1 2 and 5 B) This part of the tuft was sometimes incorporated in the thickened Bowman s capsule (Fig 5 B) When the entire tuft was affected there was usually complete occlusion of many capillaries with fibrosis and hyalinization (Figs 3 and 7 B) The degree of glomerular damage (GLI) varied from one kidney to another but for the whole group the damage was more advanced than for the other groups (Table 2)

In the interstitial tissue there was some degree of fibrosis and infiltration of lymphocytes particularly around the vessels Three of the kidneys had arteriolosclerotic changes These consisted of hyaline thickening of the walls of the arterioles in one kidney and fibrinoid necrosis with mural thrombus formation and perivascular inflammation of the arterioles and finer arteries in two

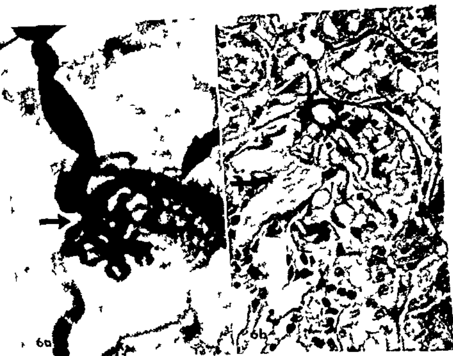


Fig 6

a Micro angiogram of a juxtamedullary arteriole glomerular unit in the right kidney of a rat in which hypertension subsided following removal of the clipped left kidney (Group I B) The arteriole forms a sharp bend at the level of the tuft (arrow) The capillary pattern of the tuft is simplified Both the pre glomerular (above the bend) and the postglomerular (below the bend) parts of the arteriole are well visualized × 255

b One serial histological section of the tissue at arrow in Fig 6 a showing the sharp bend of the contrast filled arteriole (arrow) and the fairly mildly damaged glomerular tuft Ladewig × 450

As regards JGI there was a significant difference between the left and right kidney in the rats in group I A (Table 2)

Reversible hypertension (Group I B) The injection of contrast medium failed in one of the rats in this group In all the other rats the untouched kidney had a good filling of the intrarenal vessels both in the cortex and the medulla (Fig 8) Some glomeruli however were incompletely filled or not visualized at all but this feature was much less prominent than in the rats in group I A The peritubular capillary network in the cortex showed only minor filling defects and the veins were faintly visualized or not filled at all

In the histological sections alterations similar to those in the kidneys of rats in group I A were observed but the glomerular degeneration was less advanced and extensive than in the kidneys of rats in that group (Table 2) In one of the kidneys there was a moderate degree of hyaline thickening of the walls of the preglomerular arterioles

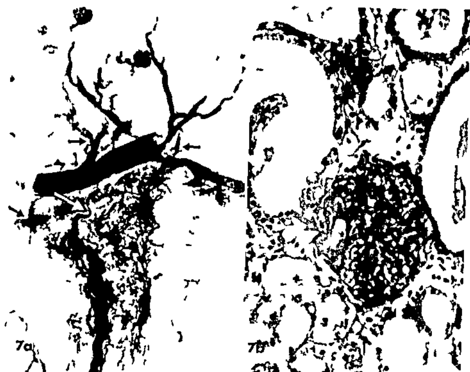


Fig 7

a Micro angiogram from the right kidney of a rat in which hypertension persisted after removal of the clipped left kidney (Group I A) There is poor visualization of the cortical vasculature (top half of picture) whereas the medullary arterioles are well visualized (bottom half of picture) Some of these are aglomerular (e g white arrow) There is venous filling in the cortico medullary area (black arrows) × 30

b One serial histological section of the tissue at white arrow in Fig 7 a The contrast filled arteriole (arrow) is seen to run through the vascular pole of a degenerated glomerulus the tuft of which is almost completely occluded Ladewig × 240

As regards JGI there was a significant difference between the left and right kidney in the rats in group I B (Table 2)

No hypertension (Group I C) The micro angiographic and histological features in the untouched kidneys of this group of rats were indistinguishable from those in the kidneys of the rats with reversible hypertension Thus glomerular lesions were encountered (Fig 9) and they were as pronounced in these kidneys as in the kidneys of the rats in which hypertension had been present but reversible (Table 2) In no kidney was there any evidence of arteriolosclerosis As regards JGI there was no significant difference between the left and right kidneys

Sham operated rats (Group II) The kidneys in these rats showed a completely normal morphology both in the micro angiograms and in the histological sections Arteriolosclerosis was absent There were practically no signs of glomerular degeneration and the tufts were



Fig 8

Micro angiogram from the right kidney of a rat in which hypertension subsided after removal of the clipped left kidney (Group I B) No major abnormalities are descernible in the vascular pattern although some glomeruli are incompletely visualized. There is no venous filling Cf Figs 4 and 7 A $\times$ 25

TABLE 2

Morphological Findings in Rats with Left Renal Artery Stenosis and Persistent Hypertension (I A) Reversible Hypertension (I B) and Normotension (I C) A Control Group of Sham Operated Rats is Included (II)

Group of rats		No of rats	Typical microang	GLI	JGI	
					left	right
	A	15	+	6 6 ± 20 1	41 7 ± 2 8	7 2 ± 1 3
I	B	14	—	12 9 ± 3 4	62 5 ± 8 4	12 2 ± 2.3
	C	8	—	15 7 ± 4 4	17 4 ± 7 9	11 9 ± 0 7
II		10	—	0 6 ± 0 3	17 5 ± 4 8	13 4 ± 6 0

GLI Glomerular lesion index in right kidney (see text)
JGI Juxtaglomerular granulation index
Index values are means ± standard error
The difference in GLI between Group II and all the other groups is significant ($P < 0\ 001$ for all groups) The difference in GLI between Groups I A and I B is also significant ($P < 0\ 05$) and that between Groups I A and I C is almost significant ($0\ 05 < P < 0\ 10$) The difference in GLI between Groups I B and I C is not significant

The difference in JGI between the left and right kidney is significant for Groups I A and I B ($P < 0\ 001$) but not for Groups I C and II

usually completely filled with contrast medium (Fig 10) Accordingly the GLI value for the right kidney of these rats was significantly lower than that of all the other groups of rats (Table 2) As regards JGI there was no significant difference between the left and right kidneys

DISCUSSION

The present investigation has shown that alterations develop in the stereoscopic micro angioarchitecture in the contralateral kidney of rats



Figs 9–10

Fig 9 Histological section from the cortex of the right kidney of a rat in which left renal artery clipping did not lead to hypertension (Group I C) One glomerulus is normal (bottom) whereas the other two glomeruli show degenerative changes with partial and complete occlusion of the tuft (top) The tubules and interstitial tissue are normal Ladewig × 170

Fig 10 Histological section of a cortical glomerulus in the right kidney of a sham operated rat (Group II) The tuft is entirely normal and its capillaries are well filled with contrast medium Cf Figs 1–3 Ladewig × 495

with unilateral renal artery stenosis These alterations differed in degree rather than in type in the kidneys of rats which remained hypertensive after removal of the clipped kidneys and in the kidneys of rats in which the blood pressure dropped to normal levels or had never been elevated The fully developed micro angiographic alterations were seen in the kidneys from persistently hypertensive rats and consisted of (i) incomplete visualization of the glomeruli and peritubular capillaries in the cortex although the filling of the medullary vessels was complete (ii) the appearance of increasing numbers of medullary arterioles the glomeruli of which were either incompletely filled or not visualized at all (iii) increased degree of filling of the venous system particularly in the juxtamedullary zone

Histologically the incomplete or absent visualization of the cortical and juxtamedullary glomeruli was due to progressive degenerative changes in these glomeruli Such glomerular lesions have previously been observed in the untouched kidney of renal hypertensive rats and

their morphogenesis has been analysed by electron microscopy (*Ben Ishay et al* 1966)

In the cortex the glomerular damage was associated with incomplete filling of peritubular capillaries This can be ascribed to the fact that the cortical efferent arterioles from which the peritubular capillaries derive are formed through the confluence of the glomerular capillaries and are devoid of any further connection with the pre glomerular vasculature This anatomic feature has been described earlier (*Ljungqvist* 1964 *Ljungqvist & Wagermark*) and is demonstrated in the present Fig 1

In the juxtamedullary zone degeneration of the glomeruli did not affect the postglomerular (medullary) filling This can be ascribed to the fact that these glomerular capillaries are lateral off shoots of continuous arterioles which descend into the medulla where they form arteriolae rectae This anatomic feature has been described earlier (*Ljungqvist* 1964 *Ljungqvist & Wagermark*) and is demonstrated in the present Fig 6

Since the passage of contrast medium from the pre to the post glomerular vasculature was rendered difficult in the cortical but not in the juxtamedullary zone of kidneys with extensive glomerular damage it is most probable that the increased degree of venous filling in these kidneys was the result of an increased passage of contrast medium through the medullary vasculature This is further supported by the observation that the veins in the juxtamedullary zone which receive the medullary venous flow, were regularly better visualized than the veins further peripherally in the cortex

Since rats with unilateral renal artery stenosis develop glomerular lesions in the contralateral kidney which induce alterations in the intrarenal vascular pattern two main questions arise By what mechanism have the glomerular lesions been produced and are the alterations in the intrarenal vascular pattern responsible for the persistence of the hypertensive state after removal of the clipped kidney? Before an attempt is made to answer these questions it is worth mentioning that *Wilson & Byrom* (1941) and *Floyer* (1951) observed arteriolosclerotic lesions in the untouched kidney of rats with persistent hypertension These lesions were believed to be produced by the hypertensive state and to be responsible for its further maintainance In the present material however only three of the fifteen rats with persistent hypertension showed arteriolosclerosis in the untouched kidney Moreover some renal arteriolosclerosis was seen in one rat with reversible hypertension These findings indicate that the persistence of hypertension can not be ascribed to renal arteriolosclerosis

Glomerular alterations unassociated with renal arteriolosclerosis have been observed in hypertensive rats with chronic pyelonephritis (*Heptinstall & Hill* 1967) and in rats with steroid induced hypertension (*Hill & Heptinstall* 1968) In both instances the glomerular

alterations were regarded as secondary to the hypertensive state *Ljungqvist & Richardson* (1969) found similar glomerular lesions in both normotensive and hypertensive rats with chronic pyelonephritis although the lesions were more severe and extensive in the hypertensive rats These findings indicate that arterial hypertension is not a necessary prerequisite for the development of the glomerular lesions Moreover similar glomerular lesions were found in the group of operated rats of the present study in which no hypertension was recorded (I C) and they were as extensive and severe in these animals as in the animals with reversible hypertension (I B)

The glomerular lesions can hardly be the result of an increased load of function on the kidney since no such lesions develop in the remaining kidney following unilateral nephrectomy in normal animals (group II) Nor can the development of the glomerular lesions be ascribed to ageing (*Beregi* 1958 *Ljungqvist & Lagergren* 1962) since the rats of group II were of about the same age as the rats of group I C although the GLI values were highly different The glomerular lesions therefore seem to be directly related to the presence of a clipped kidney This is in agreement with the findings by *Nakao et al* (1966) that extracts which are capable of producing vascular lesions without elevating the blood pressure can be obtained from clipped kidneys

It would seem quite plausible that the vascular alterations observed in the untouched kidney may induce a renal form of hypertension In the rats in which hypertension persisted following removal of the clipped kidney (I A) there were morphological evidences of an impeded cortical circulation but a well maintained and possibly even enhanced circulation through the medulla via the aglomerular juxtamedullary arterioles This situation would be capable of inducing a release of renin from the juxtaglomerular cells of the cortex and thus hypertension In the untouched kidneys of group I B the significantly milder glomerular damage had not produced alterations in the intrarenal vascular pattern that were sufficient for a renal hypertensive state to develop

It is known that unilateral renal artery stenosis when leading to hypertension also leads to an increased degree of juxtaglomerular cell granulation in the ipsilateral kidney This is generally regarded to reflect the increased production and liberation of renin Such an increase in juxtaglomerular granulation was observed in the clipped kidneys of the hypertensive rats in the present investigation (groups I A and I B) If after removal of the clipped kidney in group I A the untouched kidney had taken over the role of keeping the blood pressure high through a reduction of the cortical blood flow then an increased degree of juxtaglomerular granulation in this kidney would be expected It can be seen from Table 2 however that no such change in JGI was recorded The findings are in agreement with previous observations that the contralateral kidney in renal hypertensive rats which

is almost degranulated when the clipped kidney is present tends to reassume a normal degree of granulation when the clipped kidney has been removed (*Tobian et al* 1958) It is known however that prolongation of the hypertensive state is associated with a fall in plasma renin activity to normal levels (*Koletsky et al* 1967), although the clipped kidney remains hypergranulated (*Simpson* 1965) This suggests that various states of equilibrium between the production and release of renin may be established at a constant degree of juxtaglomerular granulation In extreme situations a low degree of granulation might actually reflect a rapid liberation of large amounts of renin and a high degree of granulation reflect a storage of renin due to a decreased liberation These possibilities have previously been emphasized by *Thurau* (1964)

SUMMARY

Hypertension was induced in a series of rats by the application of a stenosing clip on the left renal artery The clipped kidneys were removed after hypertensive periods of various lengths This resulted in normalization of the blood pressure in some rats whereas in other rats the blood pressure remained high The contralateral kidneys in these two groups of rats were examined by a combination of stereo micro angiographic and histological methods for a comparison of their vascular patterns A comparison was also made using the contralateral kidney in a number of rats in which unilateral renal artery stenosis had not resulted in hypertension and kidneys of sham operated rats

In all rats with unilateral artery stenosis glomerular lesions were seen in the contralateral kidney These lesions were particularly severe and extensive in the kidneys of rats in which hypertension persisted after removal of the clipped kidney In these rats the glomerular lesions had induced micro angiographic alterations suggestive of a relative cortical ischaemia This may constitute a morphological basis for the persistence of the hypertensive state Arteriolosclerosis was seen only in a limited number of the kidneys

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The purpose of the culture experiments to be described in this paper was to study the possible influence of an *increased proportion of large* lymphocytes and of the presence of antibody producing cells on the *in vitro* reactivity to antigen. Therefore cultures were prepared with blood cells from rabbits shortly after booster injection and stimulated *in vitro* with the immunizing antigen as in previous experiments.

MATERIAL AND METHODS

Sheep red cell immunized rabbits were bled three days after the last of three booster injections, each of 0.5 ml of 35 per cent washed SRC in saline given in the *course of one week. Leucocyte suspensions* were prepared and tested for plaque forming cells in micro incubation chambers as previously described (*Lamvik* 1968a). The leucocytes were cultured in tubes containing 2.5 × 10[6] cells in 2.5 ml of Parker's *tissue culture medium (TC 199) with 20 per cent* pooled normal rabbit serum. The tubes prepared from each rabbit were devided into two series. In one series from each rabbit 1 per cent SRC in 0.5 ml of TC199 was added to each tube as *antigen stimulator, while 0.5 ml of TC 199 without SRC was added per tube in the* other series from each rabbit. The tubes were harvested in duplicate at regular intervals. The medium was changed on the 4th and the 8th days in the cultures *incubated further. Cell counts with differential counts,* numbers of plaque forming cells and agglutinin titres were determined before culturing and at the different times of harvesting according to techniques described previously (*Lamvik* 1968a, b).

RESULTS

Numerous large lymphocytes were present in the cell suspensions prepared from booster injected rabbits and used for culturing. In four cell suspensions, the mean number of enlarged lymphocytes was 6.4 (S.D. 1.5) compared to 2.0 per cent (S.D. 0.9) large lymphocytes in eight leucocyte suspensions prepared *from non boostered* SRC immunized rabbits (Table 1). Some of the enlarged cells present in the leucocyte *suspensions* from boostered rabbits showed plaque forming ability towards SRC (Fig. 1).

In cultures without *in vitro* stimulation, the numbers of large lymphocytes showed a gradual decline and no definite blastoid change was noticed. A few plaque forming cells were found in the cultures. They showed no tendency to increase in numbers. Agglutinins *against SRC* were liberated into the culture medium in some of the non stimulated

TABLE 1

Differential Counts in Blood Cell Suspensions Prepared from Four Boostered and Eight Non Boostered Sheep Red Cell Immunized Rabbits

	Granulocytes	Monocytes	Small lymphocytes	Large lymphocytes
Boostered rabbits	23.1 ± 8.1	5.0 ± 2.6	65.5 ± 11.[illegible]	6.4 ± 1.5
Non boostered rabbits	18.7 ± 7.3	7.0 ± 3.7	72.3 ± 7.3	2.0 ± 0.9

Mean and standard deviation

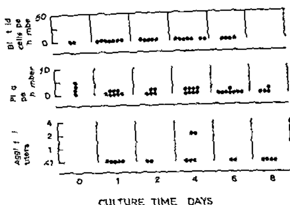


Fig 1

Non stimulated duplicate cultures prepared with blood leucocytes from four SRC immunized rabbits three days after booster injection

Figs 1–3

Variation in the numbers of blastoid cells and numbers of lytic plaques per incubation chamber plated from culture tubes harvested after different culture periods The agglutinin titres in the supernatants from each tube are also shown

cultures The titres were however never more than one dilution step higher than that found at the culture start

In the antigen stimulated cultures different results were obtained (Fig 2) Cell transformation with appearance of blastoid cells was evident after 4–6 days incubation in all culture series Concomitant with or slightly after this cells transformation plaque forming cells increased to a number several times the number of plaque forming cells present in the non stimulated cultures In the antigen stimulated cultures agglutinins against SRC were not found before the 6th day of culturing but were present in almost all culture tubes harvested after more than 6 days incubation Plasma cells were numerous after 6–8 days culturing

Fig 3 gives the variation in the numbers of blastoid cells plaque forming cells and agglutinin titres in 8 culture series prepared with cells from non boostered rabbits These data which previously have been presented separately for each culture series (Lamvik 1968b) are collected in one figure to make possible a comparison with the culture series prepared with cells from boostered animals

DISCUSSION

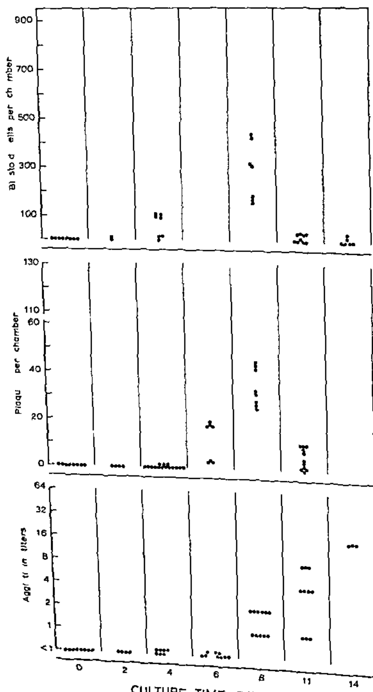
The results show that plaque forming cells are found in the peripheral blood of rabbits few days after booster injection with SRC and that small amounts of agglutinating antibodies may be liberated into the



Fig 2
SRC stimulated duplicate cultures prepared with the same cell suspensions as in Fig 1

culture medium when blood lymphocytes from SRC boostered rabbits are cultured without *in vitro* antigen stimulation These results are comparable to the observations by *Landy et al* (1964) and *Hulliger & Sorkin* (1963 1965) that antibody liberating cells appear in the peripheral blood of rabbits shortly after one injection of Salmonella antigen and after booster injection of human serum They may also be equivalent to the findings of *Wesslen* (1952) who found that rabbit thoracic duct lymphocytes collected shortly after antigen injection were able to liberate antibodies *in vitro* after 1–2 days incubation although the cells did not appear to contain antibodies when collected from the animals *Hallander & Danielsson* (1962) confirmed Wesslen s

Fig 3
SRC stimulated duplicate cultures prepared with blood leucocytes from eight SRC immunized rabbits more than six weeks after the last immunizing dose


STIMULATED CULTURES
NON BOOSTERED RABBITS
900
700
500
300
100
130
110
60
40
20
0
Plaqu per chamber
64
32
16
8
4
2
1
<1
0
2
4
6
8
11
14

finding of antibody liberation *in vitro*, but found evidence of antibody content in the cells before *in vitro* incubation as well The small numbers of plaque forming cells and minute amounts of agglutinins detected in our non stimulated cultures are probably due to antibody liberation from antibody producing cells that have entered the blood from the lymphoid organs and therefore are present in the tubes from the culture start A similar escape of antibody producing cells into the thoracic duct and peripheral blood is the most likely explanation of the above mentioned reports on antibody production and liberation by blood cells shortly after antigen injection

No blastoid transformation and no increase in numbers of antibody liberating cells occurred in the non stimulated cultures in the present experiments In antigen stimulated cultures containing blood cells from the same rabbits blastoid reaction with development of plaque forming cells and agglutinins was evident These reactions of the blood cells from booster-injected rabbits showed the same time schedule for the blastoid response and for the appearance of plaque forming cells and agglutinins as that observed in cultures containing blood cells from non boostered rabbits The same time interval (4–6 days) was found in both culture variants from culture start up to the time when a definite rise in numbers of blastoid cells set in followed by the development of plaque forming cells The similarity in the timing of the cellular and immune response suggests that the same cell type reacts towards antigen stimulation in both culture variants

The blastoid response noticed in the culture series containing cells from non boostered animals was stronger than that observed in the culture series of cells from boostered animals while no significant difference in the magnitude of the immune response in the two culture variants was found Although this may indicate that the specific immune response in relation to the degree of blastoid transformation in the antigen stimulated cultures is increased when the cell donor had been booster injected no conclusions are permissible since there was great variation in the magnitude of the response in the different culture series within each variant and since the two types of cell suspensions were not cultured at the same time

The morphological changes observed in the cultures speak in favour of the assertion that the small lymphocytes are the reacting cell type i e the precursors of blastoid cells and antibody producing cells Neutrophil granulocytes can be excluded as possible precursors because of clearly observed degenerative changes Eosinophil granulocytes are better preserved but do not show a definite morphological change Monocytes present in the cell suspensions used for culturing are probably changed to large macrophages as has also been demonstrated in skin window preparations in the rat (Vollman & Gowans 1965) No morphological evidence was found to indicate the transformation of monocytes into blastoid cells However the presence of phagocytic cells

has been found to be of importance to the immune response *in vitro* (*Lamvik* 1969). The role of phagocytic cells may be in evidence on the afferent arch of the immune reaction handling the antigen for further transfer to the precursors of antibody producing cells.

The only other possible precursors for the antibody producing cells in the rabbit blood cell cultures are small lymphocytes (resting cells) and enlarged lymphocytes which may be activated cells, which possibly may have been in recent contact with antigen in the lymphoid organs. If the latter type played a major role as reactors towards antigen stimulation *in vitro* the cellular response in cultures containing blood cells from booster injected rabbits would be expected to be stronger and possibly earlier than that in cultures from non booster injected animals, contrary to the observations made. Therefore the enlarged lymphocytes being more numerous in the blood shortly after antigen injection are probably not the cell type to react towards antigen stimulation *in vitro* presumably since they have been stimulated already *in vivo*. The only candidates left as precursor cells are the small lymphocytes.

Only a small proportion of the circulating lymphocytes will probably respond to a given antigen dependent on the magnitude of the *in vivo* immunization. When entering the blood stream they may be considered to be resting cells without ability to change into antibody producing cells without a new contact with the antigen used for priming. *In vivo* they may enter and leave the lymphoid organs (*Gowans & Knight* 1964) until they come into contact with the same antigen. When this contact occurs in an artificial milieu like a culture tube the lymphocytes apparently need 3-4 days to achieve blastoid transformation and close to one week for antibody synthesis in the blastoid cells and their progeny.

SUMMARY

Plaque forming cells and agglutinins in low titres were found in non stimulated cultures containing blood lymphocytes from booster injected sheep red cell immunized rabbits. These signs of antibody production had almost disappeared within one week's culturing. In similar cultures stimulated with antigen *in vitro*, blastoid transformation and a several fold increase in plaque forming cell numbers and agglutinin titres were noticed. The reaction was similar to that obtained in antigen stimulated cultures containing blood cells from non boostered immunized rabbits. The findings indicate that small resting lymphocytes are the cell type which responds to antigen stimulation *in vitro*.

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SPECIFICITY AND IMMUNOLOGICAL CHARACTERISTICS OF THE ANTIBODIES PRODUCED IN SHEEP RED CELL STIMULATED RABBIT BLOOD LYMPHOCYTE CULTURES

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The blastoid response with development of plaque forming cells and agglutinins against sheep red cells (SRC) which follows in vitro SRC stimulation of blood lymphocytes from SRC immunized rabbits appears to be a specific response to the stimulating antigen in contrast to the blastoid response following Phytohaemagglutinin (PHA) stimulation where no sign of specific antibody synthesis is observed (*Lamvik* 1968c) The response to SRC is dependent on *in vivo* priming of the cultured cells whereas PHA gives blastoid transformation of lymphocytes from all normal human subjects and most laboratory animals The functional meaning of the PHA response is however unknown The blastoid response which occurs in mixed lymphocyte cultures containing blood lymphocytes from two individuals or two outbred animals of the same species appears to be a better model for comparison with the antigen induced blastoid response The leucocyte antigens appear to be true histocompatibility antigens (*Terasaki et al* 1968) Therefore the mixed lymphocyte reaction may be considered an *in vitro* reaction comparable to the allograft reaction in the intact *animal* Transplantation reactions depend on the presence of small lymphocytes (*Gowans et al* 1963) which are probably also the responders to the SRC antigen in vitro

In the experiments to be reported the specificity of the blastoid response following SRC stimulation has been investigated by comparing the reactions in rabbit lymphocyte cultures *stimulated* with allogeneic cells with the cellular and immune reactions after SRC stimulation Mixed lymphocyte cultures containing *blood* cells from SRC immunized and non immunized rabbits have been checked for blastoid response and signs of anti SRC immune response and compared to the response after SRC stimulation of non mixed and mixed cell cultures

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The lytic and agglutinating agents produced in SRC-stimulated cultures behave like lytic and agglutinating antibodies produced *in vivo* as far as dependence on complement and temperature is concerned (*Lamvik* 1968b). Antibodies are best characterized by a specific reaction towards a well defined antigen used for immunization. In addition they have characteristic chemical properties. They are gamma globulins mainly of γM or γG types. Antibodies produced in the primary response in the intact animal are usually composed of γM and γG globulins while γG production predominate in the secondary immune response (*Uhr & Finkelstein* 1967). γM globulins are more potent as lytic antibodies than γG (*Wigzell et al.* 1966).

The pericellular lytic factors liberated from transformed cells are difficult to define immunochemically since no definite rise in lytic titres against SRC has been observed in the cultures. On the other hand agglutinins against SRC have been found regularly in the culture supernatants of SRC stimulated cultures after more than one week's incubation. In the second part of this paper some physico-chemical tests applied to the liberated agglutinins will be reported.

MATERIAL AND METHODS

SRC Stimulation Versus Allogeneic Stimulation

Blood cell suspensions for culturing were prepared from SRC-immunized and non immunized albino rabbits as previously described (*Lamvik* 1968a). Cells from each rabbit were cultured with or without SRC stimulation. These cultures were prepared as in previous experiments (*Lamvik* 1968a). In addition mixed lymphocyte cultures were made, each prepared with equal numbers of blood cells from each of two rabbits, SRC immunized or non immunized. Each mixed culture tube contained the same cell number as the non mixed ones, 2.5 × 10⁶ cells in 2.5 ml of medium. The mixed lymphocytes were cultured with or without SRC stimulation just as the non mixed cultures. All culture tubes were harvested after 8 days incubation with a change of medium on the 4th day. At the time of harvesting cell numbers with differential count, numbers of plaque forming cells in SRC monolayers and agglutinins against SRC were determined according to methods described previously (*Lamvik* 1968b, c).

Specificity of Reaction of Plaque Forming Cells and Agglutinins found in SRC Stimulated Cultures

Harvested cells and supernatants from SRC stimulated cultures of blood lymphocytes from SRC immunized rabbits were tested for plaque forming ability and agglutination towards rat, rabbit and human (0 Rh+) red cells according to methods described before (*Lamvik* 1968b, c). The supernatants were in addition checked for agglutination of guinea pig and goat red cell.

Treatment with 2 Mercaptoethanol

Culture supernatants from SRC stimulated cultures harvested after different culture periods were devided into two parts. One part of each supernatant was added to equal volumes of 0.2 M 2 mercaptoethanol in saline and incubated at 37° C for 30 min. Thereafter iodo acetamide was added to a final concentration of 0.11 M. The samples were then left at room temperature for 1 h and dialysed against 0.15 M NaCl over night, followed by concentration to the original volume using polyethylene glycol. Non treated and 2 mercaptoethanol treated samples were tested for agglutination of SRC.

Ad orption Elution of Agglutinins

Primarily a modification of the h at elution method of *Landsteiner & Miller* (19 5) was used Supernatants from several culture tubes harvested after identical culture periods were pooled 7 5 ml of the pooled supernatants with variable ag glutinin titres were adsorbed with 0 5 ml of packed formol treated SRC at 37 C, followed by thorough washing and elution in 0 5 ml of saline at 56 C Secondarily the adsorption elution technique of *Rubin* (1963) was used without formol treat ment of the SRC This method depends on the principle of ether destruction of red cell membranes

The eluates obtained by both methods were tested for anti SRC agglutinins and *for signs of* precipit tion by goat antirabbit gammaglobulin antiserum in agar according to the double diffusion plate technique of *Ouchterlony* (1958) The eluates were tested undiluted against four fold dilution of goat antiserum from 1/4 to 1/4096

Following adsorption and washing some SRC samples were tested for the pre nce of rabbit gammaglobulin on the cell surfaces by an antiglobulin reaction ing four fold dilutions of goat antirabbit gammaglobulin antiserum obtained om Hyland laboratories Los Angeles This goat antiserum also used in the uble diffusion tests showed on immunoelectrophoresis against pooled rabbit rum a precipitation line only in the γG region in addition to a weak line in the lbumin region The antiglobulin te t was performed on Perspex trays using 0 1 ml f antiserum dilutions and 0 1 ml of 1 per cent suspension of the adsorbed SRC. he rea tions were read from the settling pattern

ucrose Gradient Ultracentrifugation

Some culture supernatants were concentrated 3 times by the use of polyethylene lycol M wt. 20 000 Fractionation of the concentrated supernatants by sucrose radient ultracentrifugation was kindly performed by cand real Bodil Larsen Broegelmann s research laboratory

Incorporation of C^{14} Labell d Amino Acids into Immunoglobulins

C^{14} labelled l isoleucine and l lysine monohydro hloride (both with a specific ac tivity of 150 mc/mM) were obtained from The Ra liochemical Centre Amersham England 10 μc of ea h radioactive amino acid were added to 100 ml *of complete* culture medium (TC 199 with 20 per cent pooled normal rabbit serum) which contained 2 mg of non radioactive l isoleucine and 7 mg of *non radioactive* l lysine monohydrochloride per 100 ml of TC 199 The specific activities *of the radioactive* amino acids were thereby reduced to about 780 μc/mM for l isoleucine and to about 250 μc/mM for l lysine Due to trace amounts of the same *non radioactive* amino acids presumably pre ent in normal rabbit s rum the specific activities were prob ably a little below these values

SRC timulated cultures from three immunized and *two non immunized* rabbits were cultured in the presence of radioactive amino acids. *The cells* were incubated in non radioactive medium until the first change of medium after 4 days culturing Thereafter different perio ls were chosen for incubation *with* 0 3 μc of each labelled amino acid in 3 ml of complete medium per tube

At harvesting the culture media were separated from the cells and stored at −20 C until tested for anti SRC agglutinins and *for evidence* of amino acid incor poration into protein by immunoelectrophoresis followed by autoradiography Prior to immunoelectrophoresis each culture *superna ant* was dialysed against several litres of phosphate buffered saline pH 7 4 at 4 C, for 3 days with change of fluid after 1 and 2 days Thereafter the supernatants were concentrated to 1/4 volume using polyethylene glycol The *dialysed concentrated* culture supernatants contain ing all types of serum proteins from the *normal* rabbit serum in the medium were then separated by immuno electrophoresis against goat antirabbit serum using the microte hnique A constant voltage of 125 V was applied for 80–90 min Thereafter the antiserum was added and the *precipitation* reaction was allowed to o cur for 24 h in a humid chamber The preparations were subsequently washed in 1 per cent saline, dried and stained with *amido black* They were overlaid with X ray film (Kodak Dental X ray Film Ultra Speed DF 45) and exposed for *varying* periods

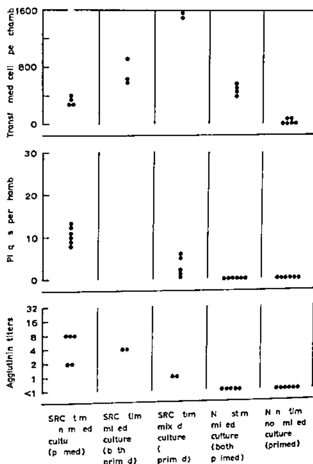


Fig 1

Numbers of transformed cells and numbers of lytic plaques per incubation chamber plated from culture tubes harvested after 8 days culturing The agglutinin titres in the supernatants from each tube are also shown Six culture variants were studied as indicated on the abscissa mixed cultures and non mixed cultures containing cells from SRC immunized (primed) and non immunized rabbits were cultured with or without SRC stimulation Each culture variant consisted of three duplicate cultures containing blood lymphocytes from three rabbits (non mixed) or three rabbit pairs (mixed cultures)

RESULTS

In three culture series the specificity of the stimulating effect of SRC antigen was investigated by comparing the functional expression of the blastoid response induced by SRC with that induced in mixed lymphocyte cultures containing cells from immunized or non immunized rabbits In addition the effect of the combined stimulation of SRC added to mixed lymphocyte cultures was examined

The results are given in Fig 1 Transformed cells developed in SRC stimulated as well as in mixed cell cultures and in the cultures stimulated with SRC and allogeneic cells The degree of cell transformation

was usually found to be higher in the double stimulated cultures than in cultures with one type of stimulation regarding the total numbers of transformed cells ($t = 2.323$ df $= 10$ $P<0.05$) as well as the numbers of large blastoid cells ($t = 2.123$ df $= 10$ $P<0.1$)

No development of plaque forming cells and agglutinins was noticed in the mixed cell cultures without SRC stimulation despite a definite blastoid response On the other hand plaque forming cells and agglutinins were found in all cultures mixed or non mixed where SRC had been added to lymphocyte from SRC immunized rabbits The immune response in SRC stimulated cultures containing cells from single SRC immunized rabbits was equivalent to the response in mixed lymphocyte cultures when both donors to the mixture were primed although the blastoid response was usually stronger in the latter culture variant In the SRC-stimulated cultures containing mixed lymphocytes from one immunized and one non immunized rabbit the numbers of plaque forming cells and the agglutinin titres were both reduced which is in accordance with the reduced number of primed cells in the cultures Mixed cultures containing cells from non immunized rabbits showed no immune response after SRC stimulation

The specificity of the pericellular lytic reaction was examined by testing two harvested cell suspensions from SRC stimulated cultures for plaque forming ability in monolayers of sheep red cells rabbit red cells guinea pig red cells and human O Rh+ red cells Numerous lytic plaques were found in the SRC monolayers while no plaques were detected in monolayers of rabbit guinea pig and human red cells

The supernatants from two SRC-stimulated cultures which gave strong agglutination of SRC were tested for agglutinating activity against rabbit guinea pig rat goat and human O Rh+ red cells with no sign of agglutination

The second part of this study was related to the character of the agglutinins found in the supernatants of SRC stimulated cultures Different physico chemical techniques were used

The agglutinin titres were determined in culture supernatants before and after treatment with 2 mercaptoethanol followed by concentration to the original volumes Culture supernatants obtained after different culture periods were tested A one step reduction in titre was observed in many supernatants However some were not affected at all and the slight reduction in agglutinating ability observed in the others is probably without significance There was no observable difference in the effect of 2 mercaptoethanol treatment on supernatants whether harvested after short or long culture periods The results indicate that the agglutinins are globulins mainly unaffected by 2 mercapto ethanol and therefore mainly composed of γG globulins

The adsorption-elution tests for further characterization of the agglutinins were done with both techniques described in the section on methods The agglutinins were easily adsorbed out of the culture

supernatants so that only trace amounts or no detectable agglutinins remained Heat elution of agglutinins from formol treated SRC in 0 85 per cent as well as in 15 per cent sodium chloride solution was tried but agglutinins were not found in the eluates and there was no precipitation in double diffusion tests against goat antirabbit gamma globulin antiserum The adsorption elution technique of Rubin according to which the red cells are broken by ether gave eluates which agglutinated SRC when supernatants containing agglutinins were adsorbed However the eluates gave no precipitation line with the goat *antiserum in agar*

In the adsorption procedure SRC were used in excess of the agglutinins present in the supernatants so that no direct agglutination of the adsorbed SRC was observed A positive antiglobulin reaction was obtained when the adsorbed cells were tested against goat anti rabbit gammaglobulin antiserum (Table 1) The results demonstrate the presence of gammaglobulins on the surface of cells adsorbed with supernatants containing agglutinins Since the antiserum used showed an immuno electrophoretic reaction towards γG globulin only the results indicate that γG has been adsorbed to the red cells

Agglutinins present in supernatants after 8 and 10 days culturing were found in fractions 5 and 6 after gradient ultracentrifugation

TABLE 1

Antiglobulin Reaction of SRC Adsorbed with Culture Supernatants and Tested Against Goat Anti Rabbit Gammaglobulin Antiserum

Supernatants used for adsorp	Dilutions of antiserum reciprocal							
	4	16	64	256	1024	4096	16384	Saline
4 days cultures	—	—	—	—	—	—	—	—
8 days	—	+	+	+	+	±	—	—
cultures	—	+	+	+	+	+	—	—
Control no adsorption	—	—	—	—	—	—	—	—

TABLE 2

Agglutinin Titres in Fractions of Culture Supernatants after Gradient Ultracentrifugation

Culture medium concentr × 3	Fractions after centrifugation									
	1	2	3	4	5	6	7	8	9	10
Before culturing	—	—	—	—	—	—	—	—	—	—
4 days culture	—	—	—	—	—	—	—	—	—	—
8 days culture	—	—	—	—	4	4	—	—	—	—
10 days culture	—	—	—	—	4	4	—	—	—	—

— titre <1



Fig 2

mmuno electrophoresis and autoradiography of concentrated supernatants from two SRC stimulated cultures incubated with C^{14} isoleucine and C^{14} lysine from the 6th to the 10th day of culturing Radioactive labelling is shown in the γG precipitation lines only demonstrating *de novo* synthesis of γG globulin in the cultures Anti SRC agglutinins were detected in dilutions 1 8 of both supernatants before concentration

(Table 2) Fractions 4 to 6 contained the γG of the normal rabbit serum used in the culture medium while fractions 1 and 2 contained small amount of γM without the presence of agglutinins The findings again give evidence of the γG composition of the anti SRC agglutinins which are liberated in the stimulated cultures

Immuno electrophoresis and autoradiography of culture supernatants from cultures incubated with radioactive amino acids showed labelling of the γG precipitation line only From each of three immunized and two non immunized rabbits cultures were examined in

TABLE 3

Incorporation of C^{14} Labelled Amino Acids into γG Globulin in SRC Stimulated Cultures Immuno Electrophoresis and Autoradiography of Concentrated Culture Media

Donors of cultured cells	Culture periods with C^{14} amino acids days		
	4–6	6–10	8–10
Immunized rabbits	±	++	+
Non immunized rabbits	—	—	—

duplicate following incubation in radioactive medium for the intervals shown in Table 3 which gives separately the pooled results of cultures from immunized and non immunized rabbits Due to the low specific activities of the radioactive amino acids in the culture medium the film exposure times had to be extended up to 10 weeks to obtain labelling of immunoprecipitation lines In cultures from immunized rabbits harvested after 6 days most preparations showed no labelling A very weak γG line was noticed in autoradiograms from two 6 days cultures in these agglutinins were also present In supernatants from cultures harvested on day 10 labelling of the γG line was found in all except one The line was however weakly labelled in several preparations particularly in those prepared from supernatants with low agglutinin titres The labelling was most definite in radiograms prepared from cultures harvested after 4 days incubation in the radioactive medium (Fig 2) In cultures containing blood cells from non immunized rabbits no agglutinins and no sign of amino acid incorporation into gammaglobulin were found

DISCUSSION

The culture experiments show that SRC stimulation of blood cells from SRC-immunized rabbits gives a cellular reaction which is functionally different from the reaction which follows stimulation with allogeneic cells Specific lytic and agglutinating antibodies directed towards SRC are found in the SRC stimulated cultures while allogeneic cells are unable to elicit this response in SRC primed lymphocytes despite a definite blastoid transformation However the blastoid response found in the mixed lymphocyte cultures may be part of another specific immune reaction possibly directed towards allogeneic antigens present in the cells from one or the other of the two donors

The combined situation of SRC stimulation in mixed cell cultures containing cells from SRC-immunized rabbits gave results which are compatible with a complete dissociation between the cellular humoral immune response to SRC stimulation and the seemingly pure cellular response due to the presence of cells with different genetic constitution Despite a stronger blastoid reaction in SRC stimulated mixed cell cultures than in non mixed ones no increase in plaque forming cells and agglutinins was found The increased numbers of transformed cells are therefore probably due to the mixed cell reaction This mixed cell reaction does not on the other hand suppress the immune reaction to SRC as no decrease in numbers of plaque forming cells and agglutinin titres was noticed

There appear therefore to be two types of lymphocytes which react with blastoid transformation in the SRC-stimulated mixed cell cultures One type previously primed against SRC responds to the added SRC and probably not towards the allogeneic leucocytes which are

present in the cultures The other type starts to proliferate because of contact with allogeneic cells without response to SRC and without subsequent anti SRC antibody production The lymphocytes in peripheral blood which have the functional ability to react towards allografts may thus belong in a cell population separate from that which contains cells *primed for antibody synthesis*

The results of the 2 mercaptoethanol treatment the antiglobulin reaction gradient ultracentrifugation and amino acid incorporation experiments all give evidence that the agglutinins which are liberated into the culture supernatants are of γG globulin type Furthermore the demonstration of labelled γG precipitation lines in the autoradiograms show that γG globulin has been synthesized de novo in the cultures It appears highly probable that the newly synthesized gammaglobulin is identical with liberated anti SRC agglutinating antibodies The results do not exclude the *production of small amounts of γM globulin* If γM were synthesized however the methods used would not be sensitive enough for its detection

One problem of interest is the relation between the lytic antibodies which cause the plaques around the transformed cells and the agglutinins found in the culture medium *In vivo* γM as well as γG globulins are formed after primary antigen injection while γG production predominates in the secondary response (*Uhr & Finkelstein* 1967) γM globulins *are more active* as *lytic antibodies* than γG in the test tubes as well as in Jerne s agar plates (*Wigzell et al* 1966) In our culture experiments agglutinins which appear to be of γG type were regularly detected in the second week of culturing while no increase in lytic antibodies was found in the same period This is in accordance with the fact that primed lymphocytes are necessary for the *in vitro* reaction which therefore seems to be equivalent to the secondary *in vivo* response However *the first sign of specific* immune reaction in our cultures is the appearance of *cells with pericellular lytic* reaction towards SRC Macroscopically visible lytic plaques *found in Jerne s* agar plates are thought to be due to liberation of γM from *lymphoid cells* (*Jerne et al* 1963) γG liberation usually gives visible plaques only *after the* addition of anti γG antibodies (*Dresser & Wortis* 1965) However *Cunningham s micro* incubation technique is more sensitive than Jerne s agar plate technique (*Cunningham* 1965) obviously due to the fact that the lytic reaction in the former occurs in a monolayer whereas the reaction in the latter is three dimensional *Some of the lytic plaques* present in the incubation chambers plated with *cultured cells* were visible with the naked eye Probably the cells would not give visible plaques on the agar plates Also in the incubation chambers the majority of the plaques were only detected microscopically It is therefore possible that the plaque *forming* cells which develop in the antigen stimulated cultures mainly *liberate γG* with relatively weak lytic activity There is however a marked variation *in* the size of the plaques

(*Lamvik* 1968c) the larger ones predominate after relatively short culture periods The presence of γM producing plaque forming cells in these short term cultures is therefore quite possible The experimental findings nevertheless indicate a type of reaction *in the culture tubes* which is comparable to the secondary response *in vivo* There are two main differences between the *in vivo* and the *in vitro* responses First the *in vitro* immune response is delayed until the 6th to 8th culture day while the *in vivo* cellular reaction with antibody synthesis is conspicuous 2–3 days after antigen *injection Secondly* the *in vitro* reaction to antigen stimulation gives a weak immune response as compared to the *in vivo* reaction The most likely explanation of these differences in timing and quantity is the obvious fact that the conditions for cell growth and cell function are inferior in the culture tubes as compared to those *in the lymphoid tissues of the intact animal* The low titres of antibodies found in the culture media may in addition be explained by low cell numbers and relatively large culture volumes

SUMMARY

The effect of SRC stimulation on cells from single immunized rabbits was compared with the effect of SRC on mixed lymphocyte cultures containing cells from SRC immunized and non immunized rabbits No sign of antibody synthesis was found in mixed cell cultures without SRC stimulation despite definite blastoid transformation *The response* to SRC stimulation appeared to be related to the number of SRC primed lymphocytes in the cultures and unrelated to any simultaneous reaction of the allograft reaction type in the culture tubes The antibodies produced showed a specific reactivity towards SRC used for *in vivo* priming and *in vitro* stimulation

The agglutinins produced in the cultures appeared to be composed mainly of γG globulin as shown by 2 mercaptoethanol treatment in *direct antiglobulin reaction* gradient ultracentrifugation and the uptake of radioactive amino acids followed by immune electrophoresis and autoradiography The latter experiments *showed de novo synthesis* of gammaglobulin *These findings* together with the fact that primed lymphocytes are necessary for the *in vitro* response indicate that the *SRC induced immune reaction* in the culture tubes is equivalent to a secondary immune response in the intact animal

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LACK OF EVIDENCE FOR ONCOGENIC OR AMYLOID INDUCING QUALITIES OF *MYCOPLASMA NEUROLYTICUM* INOCULATED INTO BALB/C MICE

By

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Received [illegible] 1 69

A connection between leukaemia and mycoplasma was first indicated by the finding of mycoplasmas in tissue cultures of biopsy specimens from leukaemic patients (12 10) Soon afterwards mycoplasma like structures were observed by the electron microscope in leukaemic tissue of both mice (6) and man (1) and mycoplasma was also isolated directly on cell free medium from leukaemic patients (14) It has furthermore been reported that tissue culture cells infected with various mycoplasma species of man may suffer irreversible alterations in their growth pattern comparable to those found in cell cultures infected with leukaemogenic viruses (16)

In previous works it has been demonstrated that plasma cell neoplasia (9) and to some extent also amyloidosis (8) are transmissible in BALB/c mice with subcellular extract of leukaemic tissue *Tully & Rask Nielsen's* finding (22) that *Mycoplasma neurolyticum* (*M neurolyticum*) is present in the BALB/c mice prompted the present experiments on the possible leukaemogenic and amyloidogenic effect of *M neurolyticum*

MATERIALS AND METHODS

Inbred BALB/c mice were kept sex segregated from 4 [illegible] of age *M neurolyticum* strain IORN 13 isolated from one of the BALB/c mice [illegible] here ([illegible]) was grown in a standard PPLO broth (3) The [illegible]0th subculture [illegible] obtained in the logarithmic growth phase and inoculated into [illegible] month old mi[illegible] Each animal received one of the following doses either 10[illegible] colony forming units (c f u) intranasally (in) in

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The authors are indepted to *J G Tully* Ph D Laboratory of Bacterial Diseases National Institutes of Health Bethesda Maryland U S A and to *R Rask Nielsen* Ph D Department of Biochemistry A University of Copenhagen Denmark for receiving the isolated *M neurolyticum*

TABLE 1

A Comparison of BALB/c Mice Kept as Untreated Controls, Inoculated with PPLO broth and Inoculated with M neurolyticum respectively

Treatment	Sex	Route of inoc*	Dose of colony forming units	Number of mice	Mean survival time months	Leukaemias Number %	Leukaemias Diagnosis †	Lung adenomas	Amyloids§ of the spleen
None	M			32	15 8 (5–23)	4 (13 %)	2 Re 1 Pl 1 Ly	0	1 3 (0–4)
Broth	M	in		15	15 3 (6–23)	0		0	1 3 (0–4)
		ip sc		13	19 4	0		0	0 8 (0–3)
M neurolyticum	M	in	10^4	20	17 7 (5–24)	1 (5 %)	1 Re	0	0 9 (0–4)
		ip sc	10^3–10^5	20	18 2 (10–23)	0		0	1 3 (0–4)
None	F			30	21 9 (9–28)	10 (33 %)	5 Re 1 Pl	0	0
Broth	F	in		15	21 0 (4–26)	5 (33 %)	2 Re 1 Pl 1 Ly 1 Gr	0	0
		ip		13	22 3 (19–26)	2 (15 %)	1 Re 1 Ly	1	0
M neurolyticum	F	in	10^4	20	21 9 (18–25)	7 (35 %)	4 Re 1 Pl 2 Ly	3	0
		ip	10^3–10^5	26	20 3 (18–27)	1 (4 %)	1 Pl	0	0

* in = intranasally ip = intraperitoneally sc = subcutaneously

† Re = reticulosarcoma (generalized) Pl = immature plasma cell leukaemia Ly = lymphocytic leukemia Gr = granulocytic leukemia

§ The amyloidosis was graded from 1 to 6 (4)

0.1 ml of broth or 10^3–10^5 c.f.u. intraperitoneally (i.p.) or subcutaneously (s.c.) in 1 ml of broth (Table 1).

A slightly modified A2 medium (20) with and without 1 per cent urea was used for cultivation of T strains of mycoplasma.

All animals were killed shortly before expected death. Lung, liver, spleen, kidney, peripheral lymph nodes, thymus and thyroid gland were taken for microscopy and stained with haematoxylin-eosin and periodic acid Schiff (PAS). In selected cases staining with alkaline Congo red was also performed. Haematocrit, serum electrophoresis (19) and white cell count were executed in connection with the autopsy. The spleen amyloidosis was graded from 1 to 6 (4).

RESULTS

Leukaemias

Uninoculated mice revealed the highest leukaemia incidence (Table 1). In mice given broth either in- or paranasally the incidence seemed independent of the presence or absence of *M. neurolyticum* in the inoculum ($0.1 < p < 0.95$). Females revealed the highest incidence of leukaemia and the longest mean survival time; furthermore, most leukaemias appeared in old mice (9).

The leukaemias showed macroscopic and microscopic manifestations as previously described for BALB/c mice (9). Most of the leukaemias were reticulosarcomas, usually of type B (7). The malignancy of tissue from five of these reticulosarcomas was confirmed by transplantation to syngeneic mice, all of which developed reticulosarcomas. Four cases of immature plasma cell neoplasias of differentiation grade III–IV (18) were found. No serum abnormalities were observed by cellulose acetate electrophoresis. Another four mice developed lymphocytic leukaemia and one mouse exhibited a granulocytic leukaemia.

In one 24-month-old mouse with reticulosarcoma, malignant cells in the lung were surrounded by big cells containing brown pigment in their cytoplasm and what seemed to be remnants of phagocytosed nuclei (Fig. 1). This mouse received *M. neurolyticum* in. Leukaemic spleen and lymph node tissue were successfully transplanted s.c. to four syngeneic recipients. In one of these mice leukaemic infiltrates in lymph nodes were interspersed with big macrophages containing dying cells.

The only other neoplasias observed were four highly differentiated lung adenomas (Table 1).

Three different subcultures of the present strain of *M. neurolyticum* were found incapable of producing rolling disease in baby mice (21).

Attempts to isolate T-strain mycoplasmas from two leukaemic mice which had been inoculated with *M. neurolyticum* and from five control mice were unsuccessful.

Amyloidosis

Amyloid was confined to males in which it occurred in nearly all spleens and sometimes in kidney glomeruli too. In both places th



Figs 1–2

Fig 1 Section of lung from 23 months old BALB/c male mouse with generalized reticulosarcoma Clusters of big cells containing brown pigment clumps in their cytoplasm surrounds an area with malignant cells PAS technique × 840

Fig 2 A greater magnification of the section shown in Fig 1 Remnants of 2 nuclei visible in the cytoplasm of one of the big pigment cells (arrow) Possibly the two nuclei are from phagocytosed malignant cells PAS technique × 2100

amyloid gave a positive staining reaction with alkaline Congo red The development of amyloid appeared unrelated to inoculation with *M neurolyticum* (Table 1) Histological evidence of chronic infection was not encountered in the mice Leucocyte counts (about 6000 per µl) and haematocrit values (about 42) were normal and nearly the same in the different groups

DISCUSSION

From the low incidence of neoplasias and other tumours in mice inoculated with *M neurolyticum* as compared to the non inoculated controls it is concluded that this micro organism was without oncogenic properties in BALB/c mice The incidences observed in the controls are in accordance with previously published findings in BALB/c mice (9 2 5)

The possibility that BALB/c mice due to neonatal infection are tolerant to *M neurolyticum* should not exclude the detection of a leukaemogenic effect by means of the present type of experiment It is well documented that AKR mice harbour a leukaemogenic virus (13) to which they are tolerant (23) and that AKR mice develop leukaemia subsequent to inoculation of this agent (15)

The fact that leukaemias associated with virus like particles may be induced by irradiation of germ free mice (24) which are free of mycoplasmas (17) also indicates that the murine mycoplasmas are not causative agents for leukaemias in mice *Girardi et al* (11) furthermore failed to demonstrate oncogenic properties of two human strains of mycoplasma (*M pneumoniae* and *M spp*) which they inoculated into hamsters

The overall slightly lower incidence of leukaemia in mice inoculated with broth irrespective of the presence of *M neurolyticum* indicated that some factor(s) in the broth might inhibit the leukaemogenic agent Solely the possibility was tested that thallium acetate present in the broth might be such a factor inhibiting a supposed T strain mycoplasma However attempts to isolate T strain from leukaemic mice were unsuccessful

The apparent phagocytosis of malignant cells observed in one mouse may be an example of a defence reaction to abnormal cells especially as the phenomenon reappeared after transplantation Infection of the malignant cells with *M neurolyticum* might be of importance for eliciting this reaction (16) which has not previously been observed in the case of our leukaemias

Occurrence of amyloidosis independent of inoculation of *M neurolyticum* and lacking histological evidence of chronic infection in inoculated mice indicate that this micro organism did not induce amyloid development It also means that *M neurolyticum* hardly is

causative of the amyloidogenic effect of subcellular extracts of leukaemic tissue from mice with plasma cell leukaemias (8)

SUMMARY

A broth culture of *Mycoplasma neurolyticum* isolated from a BALB/c mouse was inoculated into 2 month old BALB/c mice of both sexes BALB/c mice given broth alone and uninoculated BALB/c mice served as controls Leukaemia developed in about 25 per cent of the females and in 5 per cent of the males whereas amyloid occurred in most males and not at all in females The inoculation of *M neurolyticum* had no discernible effect on the development of the two diseases In one leukaemic mouse inoculated with *M neurolyticum* phagocytosis of malignant cells by big macrophages seemed to occur

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THE FLUORESCENT ANTIBODY TECHNIQUE IN THE DIAGNOSIS OF ENTEROPATHOGENIC *ESCHERICHIA COLI* WITH SPECIAL REFERENCE TO SENSITIVITY AND SPECIFICITY

By

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Received 4 II 69

The technique of immunofluorescence was first described by *Coons et al* (5 6) and was subsequently further developed by *Coons & Kaplan* (7) It has proved a valuable technical aid in various branches of microbiology including the identification of bacteria (4) It seems particularly applicable for the diagnosis of enteropathogenic *Escherichia coli* (EEC) in connexion with epidemic infantile diarrhoea Since its introduction by *Whitaker et al* (20) in 1958 in the investigation of an epidemic caused by EEC of serotype 0127 B8 the technique has been used in a number of epidemics (2 3 16 17) including Swedish out breaks (1 8 9) due to different serotypes With the aid of the fluores cent antibody (FA) technique it has been possible to demonstrate small numbers of EEC in drinking water and polluted rivers (10 11 12)

Conditions for the satisfactory working of the FA technique are high sensitivity with a low incidence of false positive reactions The diag nosis of EEC has to date fulfilled high demands in this respect but false positive reactions have been reported and are thought usually to be due to antigenic relationship with other enterobacteria (4) This has been closely studied by *Davis & Ewing* (13) who succeeded in iso lating one Salmonella one Arizona four Citrobacter six Escherichia coli one Aerobacter and one Providencia all of which gave fluores cence of varying intensity with one or more of the antibody conjugates prepared against OB antigens of different serotypes of EEC It has also been clearly demonstrated that enterococci are capable of giving strong fluorescence but these organisms are readily distinguishable from EEC bacteria by their morphology (4) On the whole the incidence of false

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positive reactions has been regarded to be too low to interfere with routine diagnostic work with infants

There are however very few data on the sensitivity and specificity of the FA technique for detection of EEC in other than infant age groups. In an epidemic of infantile diarrhoea at a children's home published by Danielsson *et al* in 1965 (9) the FA technique showed apparently false positive reactions in specimens from both staff and mothers. Fluorescence was obtained with one or not uncommonly several different serotypes despite failure to isolate the corresponding strain by conventional culture and serology.

The purpose of the present investigation was to examine the usefulness of the FA technique with particular regard to sensitivity and specificity for the detection of EEC in infants, in children at various ages and in adults. To obtain a wide age distribution the material was obtained from different institutions for infants, children and adults and also from non hospitalized individuals.

MATERIAL

The samples of faeces classified according to age of the subject and place of collection are shown in Table 1. A total of 849 samples were taken from 607 persons. The places of collection are briefly described below.

TABLE 1

Collation of the Total Number of Examined Faecal Specimens with Regard to the Ages of Patients and the Places of Collection

Age group	1 Vilan	2 UAS	3 Sater	4 Lindg.	5 Gillb.	6 Other	Total
< 1	17	23	–	–	59	5	97
1–2	38	5		[illegible]	43	3	91
2–4	46	6		24	–	6	82
4–12	67	14		50	–	19	150
16–60	119	7	142	40	48	73	429
Total	287	55	142	116	143	106	849

1 Vilan Community outside Uppsala
2 UAS Children's Department of the Uppsala University Hospital
3 Mental Hospital Sater
4 Children's Home Lindgården
5 Gillbergska *Home for Healthy Children*
6 Other Regional hospitals

Vilan. This is a small community outside Uppsala. The samples were obtained during the summer of 19[illegible], when there was an outbreak of epidemic gastro-enteritis and almost 60 per cent of the population were taken ill. Both *infants*, children and adults were infected. It was probably a waterborne outbreak caused by a leakage of sewage water into the drinking water system. The epidemic has been fully described elsewhere (1). EEC of the same type found among diseased persons were also isolated from drinking water (1). Serotype [illegible] dominated but [illegible] and O128 B12 were also isolated. Two hundred and eighty [illegible] specimens were obtained from 175 persons.

University Hospital Uppsala Fifty five samples were taken from 43 children at the Department of Paediatrics These patients were selected without regard to age or the presence of diarrhoea

Saler Hospital This is a mental hospital i central Sweden During the summer of 1965 there was a widespread epidemic of gastro-enteritis due to Salmonella tübingen To enlarge the adult series 143 samples w re taken from 140 persons

Gillbergs Home for Infants Several epidemic outbreaks of infantile diarrhoea due to various types of EEC, occurred in this home during recent years As we have mentioned in the introductory paragraphs false po itive reactions had been obtained there during a previous pidemic (9) In the present investigation 143 samples were collected from 98 infants and from the staff

Lindjarden This is a home for children over one year Altogether 116 samples were taken from 51 children and from the staff

Oth r The Bacteri logical Lab rat ry at the Institute of Medical Microbiology Univer ity of Uppsala serves a certain area and receives specimens from different ho pitals and practitioners within it Samples are as a rule ent owing to symptoms of enteritis among patients A total of 106 such samples from 100 individuals at various ages were examined

METHODS

Conventional Culture and Serology

Faecal specimens were cultured on Conradi Drigalski s agar using petri dishes with a diameter of 15 cm The plates were incubated at 37 C and 20-24 hours later typical Escherichia coli colonies were examined by slide agglutinations with rabbit anti EEC sera (OB sera) produced as described by *Edwards & Ewing* (14) Two pools each of them covering six serotypes were used for this agglutination The following serotypes were represented O26 B6 O55 B5 O86 B7 O111 B4 O119ab B13 O11 ac B11 O114 B? O119 B14 O125 B15 O126 B16 O127 B8 and O128 B12 Colonies giving positive agglutination reaction were pure cultured on glucose agar plates and tested by slide agglutination against the individual types of sera included in the pools Final confirmation was performed by tube agglutination of the O antigen with specific era according to current techniques (14) At least 25-30 colonies were tested by slide agglutination before a culture was regarded as negative

Extended Culture

The culture procedure was extended in the case of specimens giving positive results for EEC w th the FA technique but ne ative resu ts with conventional culture and serology Th se spe imens were inoculated in aerobic culture on sheep blood agar cho olate agar and sodium azide agar in anaerobic culture on sheep blood agar tryp in digest agar liver digest agar egg yolk agar and sulphite polymyxin sulphadiazine agar (SPS agar)

Five to 10 representative colonies were selected from each of the plates showing growth and suspended in saline Smears were prepared and stained in the u ual way with such c njugate as gave a positive reaction with smears prepared from the suspension of the corresp nding f ecal specimen

FA Technique

(i) *Conjugates* Twenty ml of these rabbit anti EEC sera that were used for conventi nal serology were re erved f r labelling with fluorescein isothiocyanate (FITC) or lissamine rhodamine B (RB 200) Conjugation was carried out according t curr nt techniques (15) six of the anti EEC sera wer labelled with FITC, the remaining six with RB 200 The conjugates were used as follow a) in two pools each p l containing three conjugates labelled with FITC and three labelled with RB 200 b) separately for typing of such specimens as reacted with the pools The tw p l f c njugates contained the same serotypes as the two pools used for slide agglutination

(ii) *Bact r al strains for specificity testings* The following bacterial strains were use t test the specificity of the anti EEC conjugates a) Strains of *Escherichia coli* representing the international serotypes O1 O147 They were obtained from Statens

Seruminstitut Copenhagen by the courtesy of Dr I Orskov b) Eight strains of the Alcalescens Dispar group belonging to the serotypes O1–O8 They were obtained from the National Communicable Disease Center Atlanta Ga USA c) Fifteen strains identified as anaerobic members of the tribe Lactobacillaceae were obtained from Karolinska Institutet Stockholm by the courtesy of Dr Midtvedt These bacteria had been isolated from human and rat faeces and they are associated with the metabolism of bile acids

(iii) *Handling of faecal specimens for FA tests* About 0 1–0 2 cm³ of faeces was suspended in 1 ml of physiological saline and allowed to stand for 1–2 hours at 37 C During this time coarse material sedimented Smears were made of the supernatant on well cleaned glass slides They were dried at room temperature and gently fixed by heat Staining washing and mounting of the slides were carried out in the usual way (15)

(iv) *Fluorescence microscopy* A Zeiss fluorescence microscope equipped with a dark field condensor and a high pressure mercury lamp Osram HBO 200 was used BG 12 as primary filter in combination with Zeiss 50 or 53 as secondary filters gave the best results

RESULTS

The main results of the investigation are summarized in Table 2 This shows that 64 patients were positive with the FA test for EEC while 40 patients were positive with culture and conventional serology There was complete agreement between the FA technique and the conventional procedure in 39 positive cases and in 542 negative cases Twenty five patients had positive FA tests but negative cultures while the reverse occurred in only one patient Table 2 also shows that the majority of patients positive for EEC were from the Vilan community

In Table 3 the results of the investigation are arranged according to age distribution Rather surprisingly the highest yield of positive results was obtained within the age group 2–4 years i e 31 4 per cent of this group Positive findings were relatively uncommon among infants under 1 year 5 3 per cent The corresponding figures for the age groups 1–2 years 4–12 years and those over 12 years were 16 4 per cent 21 2 per cent and 5 5 per cent respectively

Table 3 also shows the number of patients within various age groups with positive FA tests but negative cultures and those with agreeing tests and cultures It will be seen that there was complete agreement

TABLE 2

Comparison of the Diagnostic Results Achieved in 607 Persons Examined for EEC with FA Tests and Culture Procedures

	Vilan	UAS	Sater	Lunds	Cullb	Other	Total
Number of persons	175	43	140	1	98	100	607
Positive culture Negative FA	1	0	0	0	0	0	1
Positive FA Negative culture	18	0	–	2	[illegible]	1	[illegible]
Positive FA Positive culture	3	0	0	1	1	[illegible]	39
Negative culture Negative FA	121	43	138	48	[illegible]	97	542

between the FA technique and culture procedures on specimens from patients under the age of one year. With increasing age, however, there was an increasing number of patients with positive FA tests but negative cultures. In all this occurred in 25 patients. Eighteen of these were from the Vilan community. Ten of the Vilan patients disclosed positive FA tests for one or more of the serotypes that were responsible for the epidemic in this community; the positive diagnosis was considered likely as these 10 patients (1 in the age group 1–2 years, 3 in the age group 2–4 years, 4 in the age group 4–12 years and 2 in the age group 12–60 years) also had symptoms of gastro-enteritis.

TABLE 3

Comparison of the Diagnostic Results Achieved in Different Age Groups by FA Tests and Culture Procedures

Age groups	Neg FA Pos culture	Pos FA Neg culture	Pos FA Pos culture	Neg FA Neg culture	Total
	Number of persons				
1 year	0	0	3	54	57
1–2 years	0	2	7	46	55
2–4 years	0	5	11	35	51
4–12 years	1	8	8	63	80
12–60 years	0	10	10	344	364
	1	25	39	542	607

No such explanation was arrived at in the case of the FA positive results obtained in the other 15 patients, despite extended cultures of their faecal specimens and FA tests of smears of selected colonies from several different media as described under Material and Methods. The data of these patients are listed in Table 4 with regard to FA serotypes, age groups and sources of samples. The extended cultures did not reveal the type of bacteria that caused the positive results nor were any *Escherichia coli* isolated from such serotypes (O14, O90, O107) as gave moderate to weak reactions with the anti-EEC conjugate for serotype O86 B7 (see below).

A more detailed analysis of the findings from the different sources of samples is given below. Special attention is paid to *FA & culture positive results* and *FA positive but culture negative results*.

Vilan. The epidemiological circumstances of an outbreak of gastro-enteritis due to LEC in this community was described elsewhere (1 12). The findings among affected patients were dominated by EEC of serotype O111 B4 but cases with O26 B6 and O128 B12 also occurred. The incidence of positive cultures was high among children under 4 years of age but nine cases occurred among individuals aged more than 12 years. Two infants were doubly infected with serotypes O111 B4 &

O26 B6 and O111 B4 & O128 B12 *All three serotypes were demonstrated by FA tests and subsequently isolated by culture from the two infants* In five cases the findings obtained by culture changed from O111 B4 *initially to O26 B6 or O128 B12* in later specimens In all cases showing positive culture the *same serotypes* were also demonstrated by the FA technique This was also true when more than one serotype was isolated by culture

Assessing *possible false positives* the most interesting group is that comprising the 18 individuals in whom the *FA test was positive but* conventional culture negative In 10 cases analysis disclosed fluorescence for one or more of the serotypes that were responsible for the epidemic viz O111 B4 O26 B6 *and O128 B12 It was also found that* a positive FA test was usually obtained among individuals from whom the serotype in question had been isolated on a previous or subsequent *occasion* Table 4 shows however that entirely different serotypes were detected by FA testing in eight cases Thus *among infants under* one year of age there were none among children aged 1–2 years one case was FA positive for O126 B16 and O86 B7 among children aged 2–4 years two were *FA positive for O112ac B11 and O112ab B13* respectively—one of them to both serotypes and among higher age groups two were positive for O86 B7 two for O112ac B11 and one *for O127 B8*

Saler Hospital Specimens obtained in this hospital were only from

TABLE 4

Collation of EEC Serotypes Identified by FA Tests and Regarded as Possible False Positive Reactions Distribution with Regard to Institution and Age Group

Age groups	Vilan	Säter	Gillbergska	Lindgården	Other	Total number of cases
1 year	0	NSE	0	NSE	0	0
1– 2 years	O126 O86	NSE	0	0	0	1
2– 4 years	O112ac O112ab O112ac	NSE	NSE	0	0	2
4–12 years	O86 O112ac	NSE	NSE	O128 O127	0	4
12–60 years	112ac O86 *O127*	O127 O112ac	O1?7 O11?a	0	O111	8
Total	8	2	?	2	1	15

Double designations as e g O126 O86 indicate positive fluorescence for two different serotypes in the same sample

NSE No specimens examined

adults In no case were EEC isolated by culture Table 4 shows that two cases gave positive FA tests for O127 B8 and O112ac B11, respectively

Gillberg's Home for Infants During recent years several outbreaks of infantile diarrhoea have occurred here In the present investigation EEC of serotype O55 B5 were isolated from a nurse but not from any of the infants The same results were obtained with the FA technique Specimens from two nurses gave positive FA tests for the serotypes O112ac B11 and O127 B8 respectively These serotypes were not however isolated by culture

Lindgarden Home for Children EEC of serotype O128 B12 were isolated by culture and demonstrated by the FA technique from a healthy child aged 3 years Table 4 shows that two 4 year old children gave positive FA tests for O127 B8 and O128 B12 but negative cultures

Other Two positive cultures were obtained one of serotype O86 B7 from an infant and the other of serotype O55 B5 from a child aged 2 years FA tests for these serotypes were positive in both cases EEC of serotype O111 B4 were demonstrated only with the FA method in a 12 year old girl

Cross testing Against Various Enterobacteria

The results of cross testing with the international E coli O1–O147 showed that only conjugate for EEC of serotype O86 B7 gave moderate to strong reactions (2—3+) with serotype O14 O90 and O107 Furthermore conjugate for EEC of serotype O86 B7 and O112ab B13 gave slight reactions (1+) with serotype O81 as did conjugate O127 B8 and serotype O90 No cross reactions against the strains of the Alkalescens Dispar group or those of the tribe Lactobacillae were noted Preimmune rabbit sera labelled with FITC or RB 200 gave no staining reactions with any of the strains included in the specificity testings

DISCUSSION

The FA technique gave a higher diagnostic yield of EEC than conventional procedures In the case of organisms of the same EEC serotypes as those subsequently isolated by conventional culture positive FA tests were obtained in all cases except 1 out of 40 as regards the one exception culture was positive and the FA test negative The results generally agreed with those reported by others (2 3 16 17) and by ourselves in earlier papers (1 8 9)

Special attention was given to cases in which FA tests were positive but cultures negative This occurred in 25 patients which could mean that the FA method had a greater sensitivity than culturing Another possibility is that the positive FA tests were false

Detailed analysis disclosed that a positive FA test with negative

culture sometimes occurred among individuals in whom on a previous or *subsequent occasion* the same serotype was isolated by conventional procedures Based on these observations and also on epidemiological circumstances *the results in 10 of the 25 patients* with positive FA tests but negative cultures may be ascribed to a greater sensitivity of the FA method *This explanation does not seem to be valid for the* other 15 patients and their positive FA tests are therefore regarded as false Efforts to isolate EEC *were unsuccessful even though the number* of fluorescing microorganisms was often as great as that in cases in which isolation was successful *Extended cultures were in every case* unable to reveal the organisms giving fluorescence

False positive reactions did not occur in infants less than 1 year old but with increasing age there was an increasing number of probably false positive reactions Thus the relation between the number of patients with *positive FA tests but negative cultures* and those with *positive FA tests & positive cultures* were 1 8 for the age group 1–2 years 1 7 for the age group 2–4 years 1 3 for the age group 4–12 years and 1 1 5 for the age group 12–60 years These calculations are based on the figures listed in Table 3 after correction for such positive reactions as could be ascribed to the greater sensitivity of the FA method

Some serotypes were more prevalent than others among the patients in whom the positive FA tests were considered to be false positive reactions This was the case for serotypes O86 B7 O112ab B13 O112ac B11 and O127 B8 while sporadic false positive *reactions* occurred in cases of the serotypes O111 B4 O126 B16 and O128 B12 It should be pointed out that *40 per cent of the false positive* FA reactions were against antisera to either O112ab B13 or O112ac B11 *Conjugates representing these two serotypes* were not used by *Thomason et al* (18) and their association with infantile diarrhoea is *unclear The occurrence of false positive* reactions will probably increase if conjugates for other E coli *e g* O25 K11 O44 K74 and O124 B17 are *also added to the pools* used for screening faecal specimens (19) At the present we do not know which bacteria are responsible for the false positive staining reactions We have no indication that preimmune rabbit sera contributes to non specificity which is in agreement with other investigations (4)

Altogether 15 out of 607 individuals examined gave positive *FA tests* for EEC without giving conventionally positive tests If these were all false the incidence of false positives would be 2 3 per cent *An important* finding in this investigation is the fact that the most important age group infants under *2* year has shown a *low incidence* of false positive reactions (roughly 1 per cent) With increasing age the risk of false positive reactions increases *In conclusion* the FA method is both rapid and reliable in the identification of EEC and it is well suited for this purpose

SUMMARY

The sensitivity and specificity of the FA technique for detection of EEC has been studied Faecal specimens from 607 persons at different ages were examined Through a combined epidemiological and bacterio serological study it could be shown that the reliability of the FA tech nique was about 98 per cent False positive reactions occurred more frequently among older age groups but was in the most important group infants under 2 years only about 1 per cent

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STUDIES ON ANTIGEN PREPARATIONS FROM *STAPHYLOCOCCUS AUREUS*

6 *Influence of Extraction Processes on the Chemistry and Serological Properties of Polysaccharide Preparations*

By

ARNE GROV

Received 15 ii 69

Polysaccharide A (poly A) of *Staphylococcus aureus* is composed of a ribitol teichoic acid and a small mucopeptide moiety (12) The poly saccharide thus constitutes a part of the *Staph aureus* cell wall Sero logically the teichoic acid component gives a specific precipitation line on agar gel diffusion (14) Poly A also sensitizes tanned sheep ery throcytes (TSE) for agglutination by antisera to *Staph aureus*, and this ability has been found to be connected with the mucopeptide com ponent (20 8)

No separation of the teichoic acid and the mucopeptide components of purified poly A has been achieved by electrophoresis at various pH values or by ion exchange chromatography This indicates a stable linkage between the two components *Armstrong et al* (2) suggested that the teichoic acid which was extractable by cold trichloroacetic acid was associated ionically with other cell wall components How ever the existence of a covalent linkage between teichoic acid and mucopeptide now seems to be generally accepted (24 1 16) The chemical nature of this linkage has not yet been unequivocally estab lished but it is characterized by high lability to acid (16 17)

The release of poly A from crushed or intact bacteria by buffer extraction at 37 C is caused by autolytic *enzymes* (13) apparently acting on the cell wall mucopeptide (21 7) *For extraction* and sepa ration of different bacterial components the selective solubility in phenol and water has been widely *used* with Gram negative organisms Thus in *Chromobacterium violaceum* the mucopeptide structure seems to be unextractable by phenol water mixtures (25) Hence it seemed

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worthwhile to study the efficacy of this method for extraction of teichoic acid from *Staph aureus*

This paper is concerned with a comparison of the physicochemical and serological properties of two polysaccharide preparations one of which was isolated by a neutral buffer extraction at 37 C and one by phenol extraction in the cold

MATERIALS AND METHODS

Strain The *Staph aureus* type strain 1503 (15) was employed The bacteria were grown on nutrient agar plates at 37 C for 18 hrs harvested by scraping the surfaces and stored at —25 C until used

Preparation of polysaccharides Poly A was prepared according to *Haukenes* (11) except that the last fractionation on Dowex 1 was replaced by rechromatography on DEAE cellulose Desalting was performed on a Sephadex G 25 column This preparation is referred to as buffer extracted polysaccharide (BEP)

Phenol extracted polysaccharide (PhEP) was prepared as follows Forty g of wet bacteria were suspended in 40 ml of a 20 per cent solution of phenol and mixed thoroughly with a vibrator for 30 mins The suspension was then centrifuged for 15 mins at 9 000 × *g* and the phenol and water phases were separated All operations were carried out at 4 C The bacterial residue was extracted once more in the same manner and the combined water phases were precipitated at pH 1 2 by the addition of N HCl After centrifugation the pH of the supernatant was adjusted to 5 ? and 4 volumes of ethanol were added The resulting precipitate was collected by centrifugation dissolved in water and the precipitation with acid and ethanol was repeated once The final alcohol precipitate was desalted (Sephadex) and lyophilized Further purification of the alcohol precipitate was carried out on DEAE cellulose as described for BEP

Paper chromatography Preparation of acid hydrolysates and paper chromatographic analyses were carried out according to the method described earlier (10) An additional solvent system the organic phase of BuOH EtOH H O NH$_3$ d 0 88 (40 10 49 1 v/v) (2) was used for identification of sugar alcohols

Quantitative analyses Amino components present in 6 N HCl hydrolysates were determined by an automatic amino acid analyzer (Beckman Spinco Model 120 B) according to the method of *Moore et al* (18) and nitrogen phosphorus and hexoamines were measured according to the methods reported in (10)

Digestion with phosphatase Acid phosphatase from wheat germ (Type 1 Sigma) was added to an 0 1 per cent solution of polysaccharide in 0 1 M citrate buffer pH 4 8 at an enzyme/substrate ratio of ¼ (v/w) After 24 hrs at 37 C the material was precipitated by 10 per cent trichloroacetic acid (4 ml per ml of digest) and filtered The filtrates were analyzed for inorganic phosphate Controls containing polysaccharide or phosphatase only were included

Infrared spectra These were recorded on a Unicam SP 100 MK 2 instrument fitted with NaCl prism as well as grating optics The discs were made of ? 4 mg polysaccharide and 190 mg KBr

Ultracentrifugation The ultracentrifugation experiments were carried out with the Spinco Model E analytical ultracentrifuge using the Schlieren optical system The photographic plates were measured with the aid of a Leitz Wetzlar micro comparator All centrifugations were performed at ?0 C and the solvent used was 0 05 M citrate buffer pH 4 8 The diffusion coefficient was estimated from the Schlieren curve formed in the valve type synthetic boundary cell with the cup emptying at approximately 10 000 rpm The first photograph was taken immediately after the boundary had formed and four more pictures were then taken at 8 mins intervals for the diffusion calculation The speed was then increased to 59 780 rpm for the determination of the sedimentation coefficient in the standard manner The calculations were made according to *Schachman* (?3) The molecular weight was thus estimated by substitution of the diffusion and sedimentation coefficients corrected to water and zero concentration into the relationship $M_w = RTs/D (1 - \bar{V}\rho)$ Solvent density (ρ) was determined by weighing measured volumes of solvent and water in weighed capped bottles at 20 C A partial specific volume ($\bar{V}$) of 0 ?? as

found by Ghuysen *et al* (7) for a teichoic acid glycopeptide complex was used This value is consistent with those found for other polysaccharides (5)

Serological methods Production of immune sera agglutination on slide ring test precipitation agar gel precipitation absorption of antigen and antibodies indirect haemagglutination and inhibition of bacterial agglutination were carried out ac cording to the techniques used earlier (10 9 20) Inhibition of indirect haemagglu tination was tested as follows Serial twofold dilutions of the substance to be tested were made with saline in 0 25 ml volumes To each tube was then added 0 25 ml of a serum which was diluted 8 times lower than its haemagglutination titre The mixtures were incubated at 37 C for 2 hrs and at 4 C overnight and then examined for ability to agglutinate sensitized TSE

RESULTS

The BEP was eluted from the DEAE cellulose columns in the range of 0 12–0 30 M KCl *After the second run the fractions containing sero* logically active material (ring test) showed practically no UV absorp tion at 280 mμ The yield from 50 g of wet bacteria was approximately 50 mg

The PhEP was liberated from the column in the range of 0 16–0 32 M KCl The absorption at 260 as well as at 280 mμ was negligible The resulting lyophilized material was white and amorphous and the yield was approximately 25 mg

TABLE 1
Analytical Data of Buffer Extracted (BEP) and Phenol Extracted (PhEP) Polysaccharides

	BEP	PhEP
Nitrogen	3 [illegible]4 %	2 10 %
Phosphorus (organic)	5 60 %	6 [illegible]0 %
Posphorus released by phosphomonoesterase	0 14 %	0 4[illegible] %
Hexosamines (as glucosamine free base)	24 80 %	25 10 %
Glucosamine§	23 40 %	23 60 %
Muramic acid§	1 99 %	none
Lysine	0 91 %	none
Alanine	1 16 %	trace
Serine	0 36 %	none
Glycine	2 16 %	trace
Glutamic acid	1 09 %	trace
Ribitol	+	+
Galactose	trace	trace
Molecular weight	8 000	6 400

Determined as in (10)
§ Determined by the amino acid analyzer

On immunoelectrophoresis carried out with veronal buffer pH 8 6 the preparations behaved identically and showed a strong negative charge One short arc indicated homogeneity By paper chromato graphy quantities of 0 1 N HCl hydrolysates corresponding to 0 25 mg of both polysaccharides gave no spots relating to purines and pyri

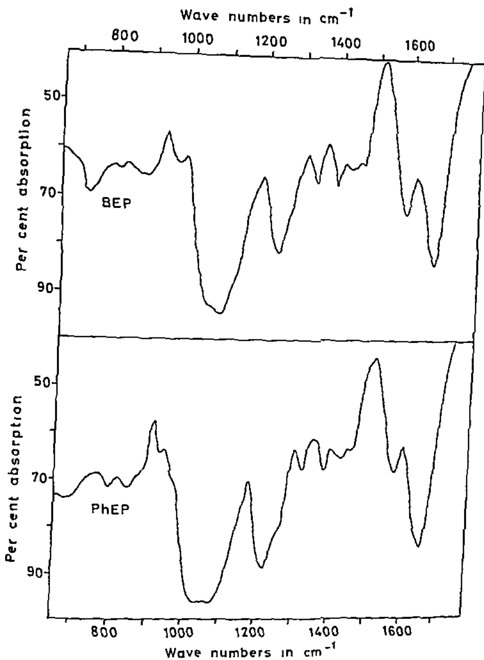


Fig 1

Infrared spectra of buffer extracted (BFP) and phenol extracted (PhEP) polysaccharides



Fig 2

Schlieren patterns of buffer extracted (BEP) and phenol extracted (PhEP) polysaccharides (4 mg per ml in 0 05 M citrate buffer pH 4 8) in the ultracentrifuge 32 mins after reaching 59 780 rpm

midines when examined under a UV lamp By this technique less than 1 μg of adenine and guanine was detected This indicates in accordance with the lack of absorbance at 260 mμ that the polysaccharide preparations are free from detectable nucleic acid contaminants

Most of the analytical data have been compiled in Table 1 In addition to the amino acids listed for the BEP trace amounts of aspartic acid threonine proline and arginine were found The trace amounts of galactose demonstrated in both preparations are probably due to a contaminant Striking differences between the two preparations were found in the content of muramic acid which was demonstrated in BEP only and in their content of amino acids In BEP the molar ratios of Mur A Lys Ala Ser Gly Glu A were 0 92 0.84 1 76 0 46 4 05 1 00

Glycerol was not detected in hydrolysates of the polysaccharides This indicated according to standards that less than 0 03 per cent could be present No amino acid was released on hydrolysis in N NH_4OH at 100 C for 5 mins and the presence of alkalilabile alanine linkages may thus be excluded (13)

Phosphate was released from both preparations on digestion with acid phosphatase In digests of PhEP free phosphate corresponding to 6 3 per cent of the total was found This gives a ratio of phosphomono ester to total phosphate of 1 15 9 For BEP the corresponding values were 2 5 per cent and 1 40

The infrared spectra of the two polysaccharide preparations show dissimilarities in the 800–700 cm^{-1} region together with some minor variations in the intensities of corresponding absorption bands (Fig 1)

In the ultracentrifuge both polysaccharides gave single symmetrical and rather slow moving sedimentation boundaries (Fig 2) No in crease of the diffusion coefficients (Dapp) with time was observed suggesting homogeneous preparations (23)

The calculations gave $S_{20\,w} = 1.34 \cdot 10^{-13}$ and $D_{20\,w} = 11.1 \cdot 10^{-7}$ for BEP and $S_{20\,w} = 1.1 \cdot 10^{-13}$ and $D_{20\,w} = 11.4 \cdot 10^{-7}$ for the PhEP with molecular weights of approximately 8 000 and 6 400 respectively

Both polysaccharides gave a single precipitation line on agar gel diffusion The lines showed reaction of identity (poly A line) The ring test titre in rabbit antiserum to strain 1503 appeared to be one dilution step higher for the PhEP than for the BEP 1/3200 and 1/1600 respectively The BEP sensitized TSE in contrast to PhEP TSE sen sitized with BEP did not seem to absorb precipitins as judged by the precipitation tests In accordance with this no decrease was observed in the precipitation titre of a solution of BEP after absorption with TSE None of the two polysaccharides inhibited bacterial agglutina tion whereas both of them inhibited indirect haemagglutination of TSE sensitized with BEP (Table 2)

TABLE 2

Serological Data of Buffer Extracted (BEP) and Phenol Extracted (PhEP) Polysaccharides Tested with 1503 Antiserum

Tests	BEP	PhEP
Ring test (dilution from 1 mg per ml)	1/1600	1/3200
Agar precipitation	poly A line	poly A line
Sensitization of TSE	+	—
Inhibition of haemagglutination (TSE s w polysaccharide A)	1/512	1/128

TSE = tanned sheep erythrocytes
Highest dilution of the antigen (from 1 mg per ml) which completely inhibited haemagglutination in the serum dilution used

DISCUSSION

The two polysaccharide preparations exhibited qualitative as well as quantitative differences in chemical composition (Table 1) The BEP contains all the components of mucopeptide and the proportions Mur A Glu A Lys Ala Gly compare well with those of the cell wall mucopeptide (7 21) Serine was present in relatively small amounts and may have replaced alanine

The PhEP seemed to be free from mucopeptide and apparently re

presents almost pure teichoic acid This suggestion is supported by serological results The precipitation line in agar gel which is due to the teichoic acid (14) was identical for both preparations BEP contains mucopeptide and accordingly sensitizes TSE for agglutination (8) in contrast to PhEP Lack of mucopeptide and inability to sensitize TSE were previously shown with pure *Staph aureus* teichoic acid (20) The PhEP is however an effective inhibitor of the agglutination of TSE sensitized with BEP This ability of the PhEP will be further discussed in a subsequent paper

A variation of 0 01 in the partial specific volume (V) changes the calculated molecular weight about 3 per cent (23) A possible difference in V of the two preparations can not however account for the observed difference in molecular weights The difference is compatible with the absence in the PhEP of a unit of N acetylglucosaminyl N acetylmuramic acid peptide According to the muramic acid and amino acid content far from all teichoic acid chains of BEP contain a mucopeptide unit In accord with this is the release of phosphate by digestion with phosphomonoesterase (Table 1) Teichoic acids have been found to contain only one terminal phosphate group which has been assumed to effect the linkage between teichoic acid and mucopeptide in the cell wall (24 16) Accordingly phosphomonoesterase has been found to have no effect on cell walls (16)

The molecular weight of PhEP (6 400) indicates a teichoic acid chain length of 14–15 units when ester linked alanine is not included This is in accord with the ratio of phosphate released by phosphatase to the total phosphate (1 15–16) In BEP the ratio of terminal phosphate to total phosphate was estimated to 1 40 Assuming the same chain length of teichoic acid as in the PhEP (14–15 units) ½ to ⅔ of the teichoic acid chains in the BEP contain bound mucopeptide This is also consistent with the amino acid and muramic acid content

Reported differences in molecular weights or in the chain length of teichoic acids may be dependent upon the isolation technique (5) It has also been suggested that difference may be a characteristic of the strain of organisms or alternatively may be due to the culture conditions (17)

Purified samples of ribitol teichoic acids extracted by trichloroacetic acid have been found to have molecular weights in the range of 4 000–5 000 (16) These figures are based on results obtained with periodate oxidation as well as on determination of terminal phosphomonoester groups Ghuysen *et al* (7) found a molecular weight of about 20 000 for a ribitol teichoic acid from *Staph aureus* strain Copenhagen prepared by enzymatic lysis of cell wall material However a trichloroacetic acid extracted teichoic acid from the same strain studied by *Sanderson et al* (22) was found to have $S_{20,w}$ = 1 13 S and a ratio of total phosphate to monoester phosphate of 14 1

The present results with PhEP closely resemble those of *Sanderson*

et al (22) and are also in reasonable agreement with the values reported by *Hay et al* (16) On the other hand this observation is not in accord with that of *Burger*, where teichoic acid extracted by trichloroacetic acid was found to be ½–⅓ the size of that extracted by phenol (5)

The infrared spectra of the two preparations show that the PhEP gives a smaller peak than the BEP at 1575 cm⁻¹ which could reflect the absence of mucopeptide in the PhEP Absorption at 1575 cm⁻¹ as well as at 1660 cm⁻¹ is due to monosubstituted amide groups (3) which are present in both acetylated amino sugars and in peptides Furthermore the small peak at 710 cm⁻¹ in the spectrum of BEP may be due to the presence of mucopeptide Chemical considerations have indicated that the linkage between teichoic acid and mucopeptide may involve a phosphoramidate bond between the terminal phosphate of teichoic acid and one of the amino groups in the mucopeptide (1 16) Infrared spectroscopic studies of compounds containing phosphoramidate linkages have shown common bands in the 750–680 cm⁻¹ region thought to be due to P-N stretching vibrations but there is no evidence for specific bands due to such linkages (4)

The linkage between teichoic acid and mucopeptide can readily be disrupted by the solvent action of aqueous phenol solutions and since there is no loss of serological activity the method could be of widespread value in isolation of teichoic acid In contrast to the trichloroacetic acid extraction procedure phenol extraction proceeds rapidly at low temperature

SUMMARY

Isolation of a polysaccharide by phenol extraction of whole *Staph aureus* bacteria is described Comparison is made physicochemically and serologically with a polysaccharide isolated by buffer extraction (polysaccharide A)

Chemical examinations indicated the absence of mucopeptide in the phenol extracted polysaccharide which had a somewhat smaller molecular weight

Serologically both preparations gave the same precipitation line (poly A) whereas only the buffer extracted polysaccharide containing mucopeptide sensitized tanned sheep erythrocytes for agglutination Both preparations inhibited indirect haemagglutination but not bacterial agglutination

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STUDIES ON ANTIGEN PREPARATIONS FROM *STAPHYLOCOCCUS AUREUS*

7 *The Component of Polysaccharide A Sensitizing Tanned Erythrocytes*

By

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Received 15 ii 69

Staphylococcal polysaccharide complexes of teichoic acids and mucopeptides sensitize tanned *sheep erythrocytes* (TSE) for agglutination in both homologous and heterologous staphylococcal antisera (21, 11 19 15 2)

Investigations reported previously (21 6 9) showed that sensitization of TSE by polysaccharide A (poly A) of *Staph aureus* is connected with the mucopeptide moiety The teichoic acid which is a specific precipitinogen (12) showed no sensitizing ability However preparations of teichoic acids have been found to be capable of inhibiting the agglutination of TSE *sensitized with staphylococcal polysaccharides* (19 2 7) This suggests that both teichoic acid and mucopeptide are necessary for sensitization and agglutination of TSE Nevertheless a mucopeptide preparation (cell wall residue) which is free from teichoic acid is strongly active as sensitizing agent (9)

This study was undertaken to clarify the role of the mucopeptide in indirect haemagglutination

MATERIALS AND METHODS

Strain The *Staph aureus* strain Wood 46 (10) was used throughout this investigation

Antigen preparations Buffer-extracted polysaccharide (poly A) and phenol extracted polysaccharide (teichoic acid) were prepared as before (7) The mucopeptide preparation used was that prepared earlier (9) from *Staph aureus* strain Cowan I

Polysaccharide A containing P^{32} (poly A P^{32}) was prepared as follows A 12 hrs culture of strain Wood 46 in 100 ml of the medium (18) (37 C) was inoculated into 2 l of fresh medium After 5 mins 500 µl of P^{32}—inorganic phosphate were added and incubation continued for a further 12 hrs with shaking The bacteria were isolated by centrifugation (30 000 × g) and washed 3 times in physiological saline The cells were extracted with buffer followed by the preparation of poly A as previously described (7)

Degraded antigens Poly A was digested with trypsin and oxidized with periodate as before (6) Oxidation was terminated by the addition of a 10 per cent solution of aqueous glucose (0 05 of total volume)

Nucleotides The preparation and isolation of UDP N acetylmuramic acid penta peptide (UDP MurNAc pentapeptide) UDP N acetylmuramic acid alanine (UDP MurNAc Ala) and UDP N acetylmuramic acid (UDP MurNAc) were carried out essentially according to the method of *Ito et al* (17) The bacteria were pre in cubated in the medium (18) (500 ml) in 1 l Roux bottles at 37 C for 10-12 hrs with continuous shaking Thereafter 500 ml of fresh medium containing penicillin G (A L Cryst) to give a total concentration of 250 μg per ml were added to each Roux bottle The incubation was continued for 90 mins during which the pH was maintained at 7 0 The bacteria were isolated by centrifugation at 30 000 $\times$ *g* and washed twice with water Nucleotides were extracted by heating an aqueous sus pension of the cells (20 ml per g of wet bacteria) at 100 C for 10 mins and were separated from the cells by sub equent centrifugation Activated charcoal (Darco G-60 Atlas Chem Ind) was then added to the extract (500 mg per g of wet bacteria extracted) and the suspension was shaken for 30 mins Before use the charcoal had been washed three times in 25 p r cent ethanol and dried at 50 C The charcoal was i olated by centrifugation washed twice with water and extracted with 50 per cent ethanol containing 0 03 M NH_4OH (1 ml per 50 mg of dry charcoal used) The alcohol extract was reduced *in vacuo* and lyophilized Nucleotides were then pre pared by fractionation on a column of Dowex-1 (formate) (16) The column (1 cm $\times$ 30 cm) was washed with water (100 ml) prior to elution with a gradient of 0-1 M ammonium formate The mixing vessel contained water (250 ml) and the reservoir M ammonium formate (250 ml pH 1 95) Fractions (5 ml) were examined for UV absorption at 260 mμ and for hexosamine content (22) The eluates within each peak were bulked desalted on a column of Sephadex G-25 and lyophilized

Sera Rabbit immune sera against *Staph aureus* strain Wood 46 and against TSE sensitized with poly A were produced as earlier (21) The latter serum is referred to as *serum TSE poly A*

Chemical analyses Hydrolysates (3 N and 6N HCl) of the prepared antigens as well as of the nucleotides were examined chromatographically according to previous descriptions (8) Amino components were assayed using an automatic amino acid analyser (7) Unhydrolysed nucleotides were examined by descending chromato graphy on Whatman No 1 and No 3 paper in the solvent systems A *Iso* butyric acid 1 M NH_4OH (5 3) (17) and B EtOH 1 M NH Ac pH 7 0 (7 5 3) (17) The spots were detected under a UV lamp Paper electrophoresis was carried out in veronal buffer pH 8 6 I = 0 1 500 V for 5 hrs

Radioactivity measurements The test materials were evaporated to dryness in circular plexiglass troughs (25 mm in diameter 3 mm high) and counted in an Electronic counter with an endwindow GM tube detector type Philips 18526 The window thickness was 1 5 mg per cm^2 A stable background of 25 cpm was obtained All samples were prepared in duplicate and the radioactivity of each trough was measured to more than 1 000 counts at least twice Samples showing small or no radioactivity as measured with the GM tube detector were checked in a Nuclear Chicago Mark I liquid scintillation counter The counter showed a stable back ground of 10 cpm The scintillating mixture contained naphthalene - 2 5 - di phenyloxazol - 2 2 - p - phenylene bis (5 phenyloxazol) - ethanol dioxan (14) Samples containing erythrocytes were decolorized by hydrogen peroxide (13)

Serological analyses Ring test agar gel precipitation indirect haemagglutination and inhibition of indirect haemagglutination were performed as reported earlier (21 7) By definition the minimum inhibitory concentration is the highest dilution of the antigen which completely inhibited haemagglutination in the serum dilution used

RESULTS

Hydrolysis showed that the poly A (buffer extracted) contained the components of teichoic acid (phosphorus ribitol glucosamine) as well as those of the mucopeptide The molar proportions of Mur A Ala Glu A Lys Gly obtained were 0 94 1 80 1 00 0 85 4 10 respectively A significant amount of serine (0 4 per cent) was also present In ad dition trace amounts of Asp A Thr Pro and Arg were detected

The absence of muramic acid and presence of only trace amounts of Ala Glu A and Gly indicated that the phenol extracted material was free from mucopeptide This preparation is further referred to as teichoic acid

Poly A and teichoic acid showed reaction of identity on agar gel diffusion In the indirect haemagglutination test poly A sensitized TSE for agglutination in contrast to teichoic acid In accordance with previous findings (6) poly A after digestion with trypsin did not sensitize TSE but retained its precipitating ability Oxidation with periodate destroyed both activities In inhibition poly A, poly A digested with trypsin teichoic acid and the mucopeptide completely inhibited agglutination of TSE sensitized with poly A Periodate oxidized preparations were inactive (Table 1) Minimum inhibitory concentrations were in the range of 1–15 µg per ml Standard N acetylglucosamine did not act as inhibitor in concentrations up to 100 mg per ml

TABLE 1

Serological Activities of Various Preparations Tested by Indirect Haemagglutination and Inhibition of Haemagglutination by Staph aureus *Wood 46 Antiserum*

Preparations	Agglutination of sensitized TSE	Aggl inhibition of TSE s w poly A
Poly A	1280	512§
trypsin digested	–	512
$NaIO_4$ oxidized	–	–
Teichoic acid	–	128
Mucopeptide (from Cowan I cell wall)	80	64
Crude UDP-Mur NAc-pentapeptide	–	16
UDP-Mur NAc-Ala	–	8
„ UDP-Mur NAc	–	8

TSE = Tanned sheep erythrocytes

Reciprocal of highest serum dilution which gave agglutination

§ Reciprocal of the highest dilution of the substance (from 1 mg per ml) which gave complete inhibition.

A typical elution curve from the fractionation of nucleotides on Dowex 1 is illustrated in Fig 1 Chromatography together with qualitative and quantitative analyses showed that the peaks A B and C contained UDP MurNAc pentapeptide UDP MurNAc Ala and UDP MurNAc respectively Glucosamine was also present in all three crude nucleotide preparations together with trace amounts of several amino acids Sugar alcohols were not detected

The crude nucleotides showed no activity on agar precipitation and ring test However all three preparations inhibited the agglutination of TSE sensitized with poly A (Table 1) The minimum concentration with inhibitory effect was in the range of 60–120 µg per ml Aliquots of the nucleotides were further retreated with activated charcoal fol

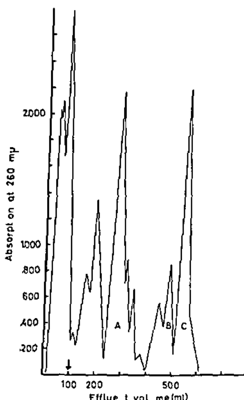


Fig 1

Separation of nucleotides from extract of penicillin inhibited *Staph aureus* strain Wood 46 by chromatography on Dowex 1 formate The arrow indicates the start of the gradient 0–1 M ammonium formate pH 4 95 The peaks A B and C contain UDP MurNAc pentapeptide UDP MurNAc Ala and UDP MurNAc respectively

lowed by gel filtration on Sephadex G 25 which removed glucosamine together with the inhibitory effect

Descending chromatography of the crude unhydrolysed fractions (A B C) was performed with the solvent system B Strips 0–2 cm which showed no colour in UV light were eluted These fractions inhibited agglutination of TSE sensitized with poly A Hydrolysis gave glucosamine and muramic acid together with the amino acids Ala Glu A Gly Lys and Ser all of which are mucopeptide components

Paper electrophoresis of the crude nucleotide preparation resulted in a wide separation of the inhibitory substance from the nucleotides The former showed slight anodic mobility and remained mainly at the origin The lowest mobility of the nucleotides was at least 8 cm These conditions also result in a rapid anodic mobility of poly A Analysis of the inhibitory substance showed it to contain almost equimolar proportions of glucosamine and muramic acid Insufficient material was available for further chemical examinations

Poly A P[1] sensitized TSE for agglutination The minimal dose for

sensitization of 0 05 ml packed TSE was approximately 10 μg, where is the minimal dose in the inhibition system used corresponded to 0 5 μg. The radioactivity measurements showed that aliquots of dilutions corresponding to 0 1 μg of poly A P32 alone or together with 0 05 ml of packed TSE gave 30–35 cpm with the GM tube detector Measurements of dried TSE only were within the background count In the scintillation counter aliquots corresponding to 0 05 μg of poly A P32 with or without erythrocytes gave 15-20 cpm above the background count Samples of 0 05 ml packed TSE were sensitized with 1 mg poly A P32 and washed 5 times in saline One of the samples was tested serologically and was found to be agglutinated by antiserum The other samples were measured for radioactivity but no radioactivity above the background was observed

Serum TSE poly A agglutinated TSE sensitized with poly A at a dilution of 1/160 but no precipitins were demonstrated This antiserum gave the same results as Wood 46 antiserum with respect to inhibition studies

DISCUSSION

The present findings agree with previous reports (21 6) and confirm that either the mucopeptide or its peptide subunit is necessary for sensitization of TSE Whether teichoic acid is attached to TSE and effects the linkage to antibody or whether this linkage is through the mucopeptide moiety has been obscure The amount of sensitizing substance linked to TSE is small and experiments in isolation of antigen from TSE have hitherto been unsuccessful

However the findings in the present studies using P32 strongly indicate that teichoic acid does not become attached to TSE by sensitization with poly A P32 No radioactivity was found in association with sensitized TSE Accordingly if present, less than 2 5 per cent of that amount of teichoic acid required to obtain inhibition of haemagglutination would be linked or adsorbed to the erythrocytes

Other experiments point to the same conclusion Firstly no precipitating antibodies were demonstrated in serum TSE poly A Also TSE sensitized with poly A did not absorb precipitins from sera In addition the precipitation titre of a poly A solution was not reduced by absorption with TSE whereas the haemagglutinogens were completely removed (21) Since only the mucopeptide component of poly A seems to be attached to TSE it must also be responsible for the serological activity

According to structural determinations of the mucopeptide (5) this component does not contain phosphorus in its basal structure The result of the present experiment with P32 is in agreement with this However mucopeptide preparations or cell wall residues which are free from detectable amount of teichoic acid always contain some phosphorus (1 9) Such preparations are active both as sensitizing (9)

and inhibiting agents To exclude the possibility that the serological activity was due to teichoic acid mucopeptide precursors were isolated Neither nucleotide precursors containing muramic acid as the sole amino sugar nor N acetylglucosamine were active whereas preparations which contained both glucosamine and muramic acid in addition to peptide inhibited agglutination of sensitized TSE This suggests that the serological activity is due to a disaccharide or a disaccharide peptide unit The structure of this saccharide may be related to those isolated from cell walls and examined by *Ghuysen & Strominger* (4) It is possible that the inhibiting substance is identical or similar to the NAc glucosaminyl NAc muramic acid pentapeptide which linked to a phospholipid carrier has been isolated from penicillin treated *Staph aureus* (23)

Inhibition studies carried out with α and β phenyl acetylglucosamine (19) have shown that both the configuration and the nature of the substituent are involved in the haemagglutination reaction On this basis the inhibitory effect of teichoic acid of *Staph aureus* is due to the glycosidically linked NAc glucosaminyl residue The results of this study show that teichoic acid does not actively participate in the indirect haemagglutination reaction The haemagglutinins and precipitins directed against poly A are undoubtedly separate antibodies The inhibition of haemagglutination by teichoic acid must be due to NAc glucosamine since this is the only constituent which is common to both mucopeptide and teichoic acid

Examination of other strains of *Staph aureus* (unpublished) have demonstrated different specificities of TSE sensitizing substances These specificities apparently depend on variations in the mucopeptide structure Thus there may be an analogy with the teichoic acids the specificities of which depend on configuration of the glucosaminyl substituents (3)

SUMMARY

The component of staphylococcal polysaccharides responsible for sensitization of tanned sheep erythrocytes (TSE) has been studied

Polysaccharide A (poly A) of *Staph aureus* sensitizes TSE for agglutination in staphylococcal antisera This reaction is also obtained with mucopeptide but not with poly A digested with trypsin periodate oxidized poly A or teichoic acid The reaction was however inhibited with poly A digested with trypsin teichoic acid mucopeptide and with preparations containing both glucosamine and muramic acid

Using a poly A preparation containing P^{32} labelled teichoic acid no radioactivity was found in association with sensitized TSE Also the fact that immune serum against TSE sensitized with poly A contained haemagglutinins but no precipitins against teichoic acid supported the view that teichoic acid is not fixed to TSE by sensitization with poly A

The mucopeptide of the cell wall is serologically active and the ability

of poly A to effect indirect haemagglutination is due to the mucopeptide moiety

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STUDIES ON ANTIGEN PREPARATIONS FROM *STAPHYLOCOCCUS AUREUS*

8 *The Substance of Crude Protein A Sensitizing Tanned Erythrocytes*

By

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Received 15 ii 69

Crude protein A (extract A) of *Staph aureus* prepared according to *Jensen*'s procedure (12) exhibited several serological activities (16 2 6) The double line appearing on agar gel precipitation against *Staph aureus* antisera is due to two different but closely related antigens which have been designated protein A and protein B (5) The precipitation in normal human sera involves protein A only Extract A also contained two sensitizing antigens (9) one of which sensitized normal sheep erythrocytes (NSE) the other tanned sheep erythrocytes (TSE) Corresponding fractions have been isolated on DEAE cellulose (9) The NSE sensitizing substance was found to be polysaccharide and could be quantitatively separated from the precipitinogens while the TSE sensitizing substance found to be peptide was not quantitatively separated from the precipitinogens

Results of cross absorption experiments (16) have indicated a structural relationship between the TSE sensitizing substance of polysaccharide A (the mucopeptide moiety) and that of crude protein A preparations Polysaccharide A as well as protein A are localized in *Staph aureus* cell walls (10) and it is tempting to assume that both contain an identical or similar peptide chain derived from the mucopeptide The intention of the present study has been to examine the validity of this assumption

MATERIALS AND METHODS

Antigen preparations Crude protein A containing the precipitinogens protein A and protein B a TSE sensitizing substance and an agglutinating substance (6) was prepared from *Staph aureus* strain Cowan I according to earlier description (9) TSE sensitizing substance free from other antigens was isolated as before (9) and chromatographed on DEAE cellulose Crude protein A without sensitizing substance was obtained by absorption with TSE (5) The preparations of polysaccharide A trypsin-digested polysaccharide A teichoic acid and mucopeptide used were those described in (8)

Antisera Rabbit antisera against the *Staph aureus* strains Cowan I and Wood 46 serum TSE poly A and serum TSE crude *protein A* were obtained as previously described (16 8 6)

Gel filtration Sephadex G-15-120 (bead size 40-120 μ) and Sephadex G-10-120 (bead size 40-120 μ) from Sigma Chem Co were used for gel filtration

Chemical analyses Preparation and qualitative paper chromatographic analysis of hydrolysates were carried out according to the methods described in (9) *Amino components* were determined using an automatic amino acid analyzer (5) N terminal amino acid residue was determined using both *dinitro fluorobenzene (DNFB)* and phenyl iso thiocyanate (PITC) as earlier described (2) The optical configuration of the amino acids glutamic acid (*Glu A*) *lysine (Lys)* and alanine (Ala) was examined using stereo specific enzymes The amino acids in the hydrolysate were separated by paper *chromatography* and eluted The concentration of each isolated amino acid was determined by measurement of colour development with ninhydrin (9 4) *and proper* amounts were then subjected to enzymatic digestions Isolated Glu A was examined using L-glutamic dehydrogenase from bovine liver *(Type II Sigma)* essentially according to the method of *Strecker* (19) A mixture of 0 5 ml of sample solution 0 3 ml of 0 1 M Tris buffer pH 8 0 and 0 2 *ml of 0 04 M diphos* phopyridine nucleotide (β DPN Sigma) was mixed with 5 μl of enzyme solution (10 mg per ml) Increase of absorbancy *at 340 mμ* was *measured* over a period of 2 hrs after addition of the enzyme Isolated Lys and Ala were examined using L lysine decarboxylase *from B cadaveris* crude acetone powder (Type I Sigma) and D amino acid oxidase from hog kidney (Sigma) respectively The methods of *Pelzer (17)* were followed The amounts of Lys and Ala not consumed by enzyme were estimated (9 4) after paper chromatography of digests together with enzyme blanks in the solvent system BuOH HAc H_2O (4 1 1)) Standard D and L Glu A L-Lys and D Ala (Sigma) were included as controls

Serological tests Ring test and agar gel precipitation indirect haemagglutination bacterial agglutination *inhibition of indirect haemag lutination and absorption* of antigens and antibodies were carried out as before (16 7)

EXPERIMENTS AND RESULTS

The sensitizing *substance of polysaccharide A and of crude protein A* cross reacted serologically (Table 1) Cross absorptions showed that TSE sensitized with polysaccharide A (TSE poly A) were able to exhaust the sera whereas TSE sensitized with crude protein A (TSE crude protein A) *did not give complete absorption of agglutinins to* TSE poly A Thus there seems to be a serological difference between the two substances sensitizing tanned erythrocytes

TABLE 1

Haemagglutination Titres (Reciprocal Values) in Sera after Cross Absorption with TSE Poly A and TSE Crude Protein A

Sensitized sheep erythrocytes	Cowan I antiserum absorbed with		Wood 46 antiserum absorbed with	
	TSE poly A	TSE crude protein A	TSE poly A	TSE crude protein A
TSE poly A	– (80)	80	– (20 480)	80
TSE crude protein A	– (20 480)	–	– (80)	–

TSE poly A and TSE-crude protein A Tanned sheep erythrocytes sensitized with polysaccharide A and crude protein A respectively
In brackets Reciprocals of the titres before absorption

TSE poly A and TSE crude protein A were agglutinated by their respective antisera. Cross reactions were also obtained but the titres appeared to be higher in the homologous sera than in the heterologous sera 1/80 and 1/40 respectively. Precipitins or agglutinins toward whole live staphylococci were not detected in these sera.

TABLE 2
Haemagglutination Inhibition

Inhibitors	TSE-crude protein A in Cowan I antiserum	TSE poly A in Wood 46 antiserum
Crude protein A	1024	32
Polysaccharide A	32	1024
Teichoic acid	No inhibition	512
Trypsin digested polysaccharide A	No inhibition	512
Mucopeptide	64	64
Crude protein A from which sensitizing substance is removed	64	>2

The figures given are reciprocals of the highest dilution of inhibitors (from 1 mg per ml) which gave complete inhibition.
TSE crude protein A and TSE-poly A: see Table 1.

Crude protein A inhibited agglutination of TSE poly A in Wood 46 antiserum at a dilution corresponding to a concentration of 30 μg per ml and agglutination of TSE crude protein A in Cowan I antiserum at a concentration of approximately 1 μg per ml (Table 2). With polysaccharide A the corresponding inhibitory concentrations were 1 and 30 μg per ml respectively. The mucopeptide preparation inhibited both systems similarly, the minimal inhibitory concentration being approximately 15 μg per ml.

In contrast to polysaccharide A, neither teichoic acid nor trypsin digested polysaccharide A inhibited agglutination of TSE crude protein A.

A crude protein A solution was absorbed with TSE until all sensitizing substance was removed as indicated by its inability to sensitize TSE. This absorbed solution still inhibited agglutination of TSE crude protein A at a concentration of approximately 15 μg per ml (Table 2).

Hydrolysates of isolated TSE sensitizing substance have earlier been found to contain Lys, Gly, Glu, Ala, Ser and trace amount of Asp A (9). Chromatography of crude protein A (9) was repeated in the present study and the isolated TSE sensitizing substance was further rechromatographed on DEAE-cellulose. The serologically active material was recovered in the second 10 ml fraction indicating no adsorption to the column material.

On gel filtration using Sephadex the TSE sensitizing substance was eluted in the imbibed volume through G 15 (exclusion limit 1500).

whereas it was eluted in the void volume through *G 10* (exclusion limit 700). Teichoic acid Mw 6000–7000 was used to determine the void volumes. The serologically active materials were located using the ring test for teichoic acid and indirect haemagglutination for the TSE sensitizing substance.

Before hydrolysis the sensitizing substance was examined for free NH group reactions. Chromatographic examination of DNP derivatives showed the presence of DNP Ala and trace amount of ε-DNP Lys. With PITC only PTH Ala was detected. Thus Ala is the only N-terminal residue.

The TSE sensitizing substance was then subjected to further chemical analyses. No phosphate was detected and the 3 N HCl hydrolysate contained no reducing sugars, sugar alcohols or amino sugar components. Amino acid analysis gave Ala, Glu A, Lys, Gly and Ser. Thus Asp A is not a component of the sensitizing substance as reported earlier (9). The molar ratios of Ala : Glu A : Lys : Gly : Ser were found to be 1.8 : 1.0 : 0.9 : 2.9 : 0.3. A considerable amount of NH_3 was also detected (90 mol per mol of Glu A), the origin of which is unknown.

The results show that the sensitizing substance is a peptide. The molar ratios of the component amino acids coincide well with those of the mucopeptide moiety present in polysaccharide A (8).

One hundred mμmole of standard L-Glu A incubated with L-glutamic dehydrogenase in the presence of excess DPN increased the absorbancy at 340 mμ due to reduced diphosphopyridine (DPNH) from 0 to 0.56. Maximum was reached within 40 mins. Standard D-Glu A (100 mμmole) gave no detectable increase in absorption. Isolated Glu A (140–150 mμmole) gave a small increase 0 to 0.05. A similar test with approximately 150 mμmole Glu A isolated from polysaccharide A (the mucopeptide moiety) showed an equal increase of absorption at 340 mμ. Thus most of the isolated Glu A had the D-configuration. Isolated Lys (100 mμmole) was completely consumed by L-lysine decarboxylase. Isolated Ala was only partly consumed by the D-amino acid oxidase. Although the contaminated enzyme preparation reduced the accuracy of the quantitative determination, approximately 40 per cent of the isolated Ala was estimated to be D-Ala.

DISCUSSION

Cross reactions and inhibition experiments showed that crude protein A and polysaccharide A have a common immunological determinant. No sugar was detected in the isolated TSE sensitizing substance; the common determinant therefore is apparently a peptide. This is confirmed by the fact that neither teichoic acid nor trypsin digested polysaccharide A inhibited agglutination of TSE crude protein A. Furthermore both sensitizing and inhibiting abilities of crude protein A were destroyed by proteolytic enzymes (2).

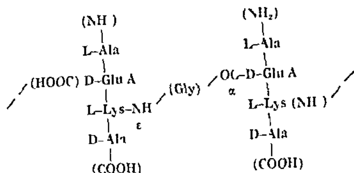


Fig 1

Proposed two dimensional basal structure of the isolated TSF sensitizing substance.

With few exceptions natural peptides and proteins are composed of L isomers of amino acids The presence of D isomers of Glu A and Ala and sometimes Asp A (18) in the cell wall peptide is unique and distinguishes this peptide from other naturally occurring substances The amino acid sequence of the *Staph aureus* cell wall peptide has been shown to be L Ala D Glu A L-Lys D Ala (18) The linkage between Glu A and Lys is effected through the γ COOH group of Glu A and the α NH group of Lys (15) L Ala the N terminal residue is linked through its NH group to the COOH group of muramic acid (18) A further character of the cell wall peptide is the unique cross linkage structures revealed by the unusual behaviour of cell walls or mucopeptide to DNFB i e relatively few ε NH groups of Lys react with DNFB (14 18) This has been found to be due to a cross linkage between the ε NH group of Lys in the one site and an available C terminal residue on the adjacent peptide chain in the other site in *Staph aureus* by pentaglycyl peptide bridges (14 1 15) Thus staphylococcal mucopeptides in general (18) contain Glu A Lys Ala Gly in the ratios of 1 1 2 5 respectively

Almost all the content of Glu A of the isolated TSE sensitizing substance (above 90 per cent) was D Glu A while both D Ala and L-Ala were present Ala was the only N terminal residue detected These data together with the amino acid composition and their molar proportions strongly suggest that the TSF sensitizing substance is a cell wall peptide and similar to that of polysaccharide A (8) A common peptide group thus explains the cross reaction between polysaccharide A and crude protein A in indirect haemagglutination Furthermore in polysaccharide A the peptide unit is linked to an amino sugar component which induces additional specific antibodies (8) and explains the serological difference observed

The Sephadex filtration experiments may indicate a molecular weight of the isolated substance between 700 and 1500 Since the calculated molecular weight for Glu A_1 Lys_1 Ala Gly_5 is 703 the substance may be composed of 2 tetrapeptide units (L-Ala D Glu A L-Lys D Ala) cross

linked through a pentaglycyl peptide bridge (Fig 1) Such a structure and size (Mw approximately 1100) also explains the relatively low per centage of Gly demonstrated

The isolated TSE sensitizing substance bears a striking resemblance to the *Staph aureus* cell wall peptide polymer studied by *Hisatsune et al* (11) These authors concluded that the α COOH of Glu A was ami dated ($CONH_2$) and that the cross linkage by pentaglycyl peptide bridges was effected through the ε NH group of Lys and the COOH terminal group of D Ala Results from other studies on *Staph aureus* mucopeptide have led to the same conclusion (1)

The behaviour of the TSE sensitizing substance on anion exchange chromatography indicates a neutral or weakly positively charged com pound The great amount of NH_3 revealed by the amino acid analyzer makes it impossible to decide whether or not an amide structure is present However carboxypeptidase digested the sensitizing substance of crude protein A (2) and also that of polysaccharide A (3) Since the precipitinogen protein A was not destroyed endopeptidases were not present Consequently a C terminal residue must be present in both the isolated TSE sensitizing substance and in polysaccharide A Providing the structure described above viz tetrapeptides cross linked by pentaglycyl bridges the bridge is most likely effected through the α COOH group of Glu A Thus D Ala should be a C-terminal residue in agreement with the structure originally proposed by *Mandelstam & Strominger* (13) Incomplete digestion of the peptide of polysaccharide A with carboxypeptidase (3) may indicate the existence of both types of linkages This may depend on whether the two tetrapeptides linked are derived from the same amino sugar chain of the mucopeptide or whether they occur on two adjacent sugar chains The possibility that there may be at least two types of glycine cross bridges has also been suggested by *Ghuysen et al* (1)

The cell wall peptide polymer of *Hisatsune et al* (11) having a greater molecular size than the present sensitizing substance has been found to be precipitated by *Staph aureus* antiserum The molecular size of the sensitizing substance is apparently too small to give pre cipitation

Agglutination of TSE crude protein A and TSE sensitized with the isolated substance was inhibited with the pure precipitinogen protein A indicating common determinant groups between protein A and the sen sitizing substance Recently *Live & Rann* (13) have reported that aldehyde tanned erythrocytes adsorb the precipitinogen protein A However more data are needed before further conclusions can be drawn on a possible connection in structure and function

The basal structure of the *Staph aureus* cell wall mucopeptide thus contains two serologically specific determinants viz the amino sugar chain (8) and the peptide units linking the sugar chains together Small variations in these structures from strain to strain are expected to give

rise to different specificities which should be revealed by cross reaction experiments

SUMMARY

The substance of crude protein A preparations sensitizing tanned sheep erythrocytes for agglutination has been characterized as a cell wall peptide Acid hydrolysates contained D Glu A, D and L-Ala L-Lys Gly and small amounts of Ser Ala was found to be the only N terminal residue In gel filtration the sensitizing substance was eluted in the imbibed volume through Sephadex G 15 (exclusion limit Mw 1500) and was eluted in the void volume through Sephadex G 10 (exclusion limit Mw 700)

It is concluded that the sensitizing substance of crude protein A is identical in structure to the peptide unit of polysaccharide A i e to the peptide subunit of the mucopeptide The common peptide unit thus explains the serological cross reactivity of these preparations in indirect haemagglutination The amino sugar chain of the mucopeptide exhibits an additional serological specificity

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GROWTH OF RESPIRATORY SYNCYTIAL (RS) VIRUS IN HEp-2 CELL CULTURES

Difference in Effect of Proflavine and Actinomycin D

By

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Received 26.II.69

It has been described previously that proflavine accelerates the development of the cytopathic effect (CPE) and increases the amount of complement fixing antigen produced by RS virus in HEp-2 cell cultures (8). The effect of proflavine and of actinomycin D on the production of infectious RS virus is reported in the present paper and for comparison the effect of these compounds on the production of infectious adeno (type 3) virus in this cell line is also described. The findings presented give further support to the conception that the multiplication of RS virus takes place in the cytoplasm independently of the DNA directed RNA and protein synthesis of the host cell. The difference in the observed effect of proflavine and actinomycin D on the multiplication of the two kinds of viruses mentioned above can probably be explained by the different sites of action of these compounds on the host cells (18).

MATERIAL AND METHODS

Tissue culture. HEp-2 cells were grown in 5 cm Petri dishes and in 150 × 12 mm tubes as described previously (9). For acridine orange (AO) experiments HEp-2 cells were grown on coverslips in Leighton tubes. Maintenance medium was in all experiments instant tissue culture powder medium 199 (with Hank's salts and l-glutamine but without bicarbonate) supplemented with 2 per cent chicken serum and 0.17 per cent bicarbonate.

Harvesting and titration of virus. Cell fractions of virus infected *monolayers* of HEp-2 cells in Petri dishes were harvested in phosphate buffered saline as described previously (9). Infectivity titrations were carried out in tube cultures of HEp-2 cells using $10^{0.7}$ (1:5) *dilution increments* and three tubes per dilution and the number of fifty per cent tissue culture *infective* doses (TCD 50) were calculated according to the method by *Reed & Muench* (1938). The *standard* deviation of the titre determined by this method *is 0.3[illegible] in log 10* units, as determined by the *method* given by *Davis et al.* (1967) which means that the titres obtained should differ *more* than 1.41 (in log 10 units) to show significant differences with 95 per cent confidence limits. With the method *of titration* used in the present paper, the *titres* should *differ* more than two dilution steps before significant differences are obtained.

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Virus strains employed The Long strain of RS virus adapted to growth in HEp-2 cells through more than 40 passages in this cell line and an adeno type 3 strain (kindly supplied by the Enterovirus department of the State Serum Institute Copenhagen) passed seven times in HEp-2 cells before being employed in the experiments to be described

AO staining of HEp-2 cells The technique described by Pearse (1960) was employed with the following modifications A citrate phosphate buffer with a pH of 3 0 and a staining period of 45 minutes was used

Coverslips with HEp-2 cells stained with AO and mounted on slides were viewed under a Leitz microscope employing a Mercury high pressure vapour lamp HBO 200 a Leitz BG12 exciter filter and a Leitz K530 barrier filter

Compounds used Proflavine hemisulphate was obtained from T & H Smith Ltd Edinburgh Actinomycin-D was kindly supplied by Merck Sharp & Dohme and Acridine Orange N (AO) was obtained from Edward Gurr Ltd London

Concentrations of proflavine and of actinomycin-D employed A concentration of 2 μg of proflavine per ml or of 0 08 μg of actinomycin D per ml of maintenance medium caused some rounding up of the HEp-2 cells after incubation of the tissue cultures for 48 hours in the dark at 35 C In all experiments to be reported a proflavine concentration of 1 μg per ml or an actinomycin D concentration of 0 04 μg per ml were used and with these concentrations no visible degeneration of the noninfected control cultures was observed during the experimental periods employed The effect of proflavine or of actinomycin D on the cell number was followed by counting the cells in a haematocytometer after trypsination of the monolayers The cell number of noninfected cultures supplemented with the last mentioned concentrations of these compounds always varied less than twofold (with one exception to be described) in comparison with control cultures (duplicate cultures were counted) when the counting was performed at the end of the experimental period According to earlier findings (10) a less than twofold variation in the cell number does not influence the titres of infectious RS virus obtained in HEp-2 cell cultures

EXPERIMENTAL

Effect of Supplementing the Maintenance Medium with Proflavine before, during, or after the Absorption of RS Virus to HEp 2 Cultures

Preliminary experiments showed that proflavine only exerted its accelerating influence on the virus induced CPE when a multiplicity of infection number of TCD 50 per cell (MOI) lower than 0 1 was employed Fig 1 shows the results of two experiments where a MOI of 0 05 or a MOI of 0 005 were employed with an absorption period of 3 hours The titres shown reveal the differences between the titres (log TCD 50_P per ml of cell fraction) obtained in cultures with proflavine and the titres (log TCD 50_C per ml of cell fraction) obtained in parallel cultures without proflavine In the experiment with the MOI of 0 005 the cell fractions were harvested 48 hours after infection (PI) and in the experiment with the MOI of 0 05 the cell fractions were harvested 24 hours PI Fig 1 shows that proflavine gives a significant increase in infectious virus with the lower MOI and only when it is added to the cultures after the absorption period In both experiments an acceleration of the cytopathic effect was seen in the cultures supplemented with proflavine but most pronounced in the experiment where a MOI of 0 005 was employed

Fig 2 shows the results of an experiment where proflavine was added

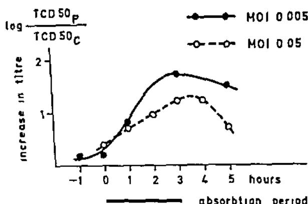


Fig 1

The effect of adding proflavin to HEp-2 cultures at different hours before during or after the 3 hour absorption period of RS virus The figure shows the increase in titre of infectious virus obtained in the proflavine containing cultures The results of two experiments are shown one with a MOI of 0 05 and the other with a MOI of 0 005 Abscissa Time (hours) of addition of proflavine (1 μg/ml) Ordinate Log_{10}TCD50 per ml of cell fraction of cultures containing proflavine (P) minus log_{10}TCD50 per ml of cell fraction of control cultures (C) without proflavine

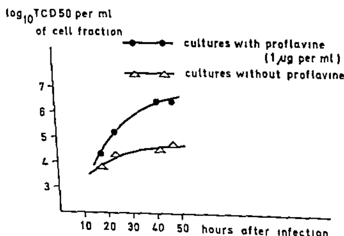


Fig 2

Increase in virus yield produced by adding proflavine (1 μg/ml) to RS virus infected HEp-2 cultures (MOI 0 00) immediately after the hour absorption period

to a number of HEp 2 cultures just after they had been infected with RS virus (absorption period 3 hours MOI 0 005) At various intervals after infection (18 24 42 and 48 hours) the amount of infectious virus was determined in duplicate cultures both with and without proflavine Fig 2 reveals that the added proflavine gives a significant increase in the yield of infectious virus in the cell fractions titrated two days after infection The experiment shown in Fig 2 was repeated with a MOI of

0 05 but the added proflavine could not produce a significant increase in virus yield with this MOI

Effect of Supplementing the Maintenance Medium with Proflavine before during, or after the Absorption of Adenovirus to HEp-2 Cultures

A number of HEp 2 cultures (in Petri dishes) were infected with adeno (type 3) virus (MOI 0 005 absorption period 4 hours) and at various intervals before during or after the absorption period the maintenance medium of duplicate cultures was supplemented with proflavine Fortyeight hours after infection the virus yield was determined in the cell fractions of cultures both with and without proflavine No significant differences between the adenovirus titres measured could be found even though proflavine decreased the titres approximately one log 10 unit when added to the cultures before or during the absorption period

Effect of Pretreating Hep-2 Cultures with Proflavine or Actinomycin D before Infection with RS Virus or Adenovirus

A number of monolayers in Petri dishes were incubated with proflavine or with actinomycin D for 24 hours prior to infection with either RS virus (MOI 0 05 absorption period 3 hours) or adenovirus (MOI 0 05 absorption period 4 hours) Before the absorption of virus the cultures were washed three times in maintenance medium and proflavine or actinomycin D was not included during the rest of the experiment Fortyfour hours PI the virus yield was determined in the cell fractions of the different cultures The results are shown in Table 1 which indicates that pretreatment of the cells with actinomycin was able to decrease the infectious titre of both kinds of viruses concerned while proflavine did not have this effect Determination of cell numbers in noninfected cell cultures at the end of the experiment showed that pretreatment with proflavine had produced a 30 per cent decrease and pretreatment with actinomycin D had produced a 50 per cent decrease

TABLE 1

Effect of Supplementing the Maintenance Medium of HEp-2 Cultures with either Proflavine (1 μg per ml) or Actinomycin-D (0 04 μg per ml) for 24 Hours before the absorption of RS Virus (MOI 0 05) or of Adenovirus (MOI 0 05)

Virus investigated	Log$_1$ TCD 50 per ml of cell fraction 44 hours PI Pretreatment of HEp-2 cells for 24 hours with		
	—	Proflavine 1 μg per ml	Actinomycin D 0 04 μg per ml
RS	6 6	5 9	4 7
Adeno	5 1	4 8	2 7

in the cell number compared with the cell number in cultures without these compounds When the decrease in virus yield caused by actinomycin D (Table 1) is evaluated the decrease in cell number in non infected cultures caused by actinomycin must of course be taken into consideration

Effect of Adding Actinomycin D to the Maintenance Medium before, during or after Absorption of RS Virus

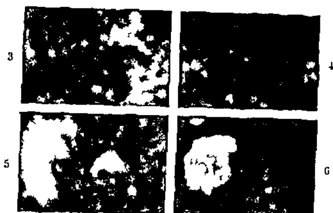
A number of monolayers (in Petri dishes) were infected with RS-virus (MOI 0 5 absorption period 3 hours) and actinomycin D was added (to duplicate cultures) one hour before together with just after and three hours after the absorption of the virus Cell fractions were harvested and titrated for virus yield 24 hours PI but no significant differences in the titres obtained were found The experiment was repeated with a MOI of 0 005 the cell fractions were harvested at 48 hours PI but no differences in the titres obtained were seen

Effect of Adding Actinomycin D to the Maintenance Medium at the Beginning of the Absorption Period of Adenovirus

A number of monolayers in Petri dishes were infected with adeno virus (MOI 0 5 and absorption period 4 hours) and maintenance medium was supplemented with actinomycin D from the start of the absorption period until the cell fractions were harvested and titrated 44 hours PI The virus titre in the cultures containing actinomycin D was approximately 3 log 10 units lower than the corresponding titre in the cultures without this compound

AO Staining of RS Virus Infected Cultures Supplemented with Actinomycin D

Confluent monolayers of HEp 2 cells on coverslips in Leighton tubes were infected with RS virus employing a MOI of 0 05 and following an absorption period of three hours the maintenance medium was supplemented with actinomycin D in a concentration of 0 04 μg per ml Fig 3–6 show the effect of actinomycin D on the distribution of AO stainable RNA and DNA following incubation for approximately 68 hours of noninfected (Fig 3 and 4) and infected (Fig 5 and 6) monolayers Fig 3 is a normal monolayer without actinomycin Fig 4 shows a loss of the major part of cytoplasmic RNA and of nucleoli caused by incubation with actinomycin Fig 5 is an infected monolayer without actinomycin (part of a syncytia is seen to the left in the picture) Fig 6 shows the effect of actinomycin D on an infected monolayer An accumulation of RNA is seen in the virus induced syncytium and the rest of the cells (probably noninfected) have an appearance like those in Fig 4



Figs 3–6

HEp-2 cultures noninfected (Figs 3 and 4) and RS virus infected (Figs 5 and 6) AO staining 68 hours PI Actinomycin-D (0 04 µg per ml of maintenance medium) added immediately following an absorption period of three hours to some of the infected cultures (represented by Fig 6) and at the same time to some of the noninfected cultures (represented by Fig 4) No actinomycin-D added to cultures represented by Fig 3 and Fig 5

DISCUSSION

Some differences in the effect of actinomycin D and proflavine on non infected cells and on virus infected cells have been described Actinomycin D binds to host cell DNA thereby selectively inhibiting biosynthesis of cellular RNA (14) Proflavine binds to both host cell DNA and RNA preferably to DNA (18) and should in this way have a more direct inhibiting influence on cellular protein synthesis possibly by binding to transfer RNA (21) Proflavine causes primarily a destruction of cytoplasmic structures (3) and produces also a characteristic change in the structure of the nucleoli (16) Even low concentrations of actinomycin D can inhibit cell growth and cause a decrease in RNA both in nuclei and in cytoplasm (15)

Actinomycin D does not inhibit the growth of poliovirus in HeLa cells (15) but another small RNA virus foot and mouth disease virus is inhibited by proflavine when grown in swine kidney cells (5) Actinomycin D produces a shorter latent period of multiplication of Sendai virus in chick embryo cells (6) but proflavine inhibits the production of Sendai virus in HEp 2 cells (2) Some of the observed differences in the effect of actinomycin D and proflavine are probably caused by the different test systems employed but some are probably caused by the binding of proflavine to host cell RNA as mentioned above

In the present paper an enhancement of growth of RS virus by proflavine has been described but enhancement of growth of this virus by actinomycin D could not be produced with the concentration employed and the MOI used Furthermore actinomycin D added to HEp 2 cultures infected with either adenovirus or RS virus only caused inhibition of

adenovirus Wilcox & Ginsberg (1962) reported inhibition of adeno (type 5) virus growth by proflavine in KB cells employing a MOI of 10 or 0.01. In the present report no significant inhibition of adeno (type 3) virus growth by proflavine in HEp 2 cells could be achieved employing a MOI of 0.005.

Actinomycin D and proflavine cause essentially the same effect of the influenza subgroup and on the Newcastle disease virus (NDV) subgroup of myxoviruses (19). Both compounds inhibit the growth of fowl plague virus in concentrations which do not influence the growth of NDV (4, 18). It has been difficult to classify RS virus with the members of the NDV subgroup (20) and it does not belong in the influenza subgroup. This aspect might explain why actinomycin D and proflavine do not have a similar effect on RS virus.

Several examples of enhancement of the growth of myxoviruses and arboviruses by actinomycin D but not by proflavine have been described (1, 4, 8, 22, 23, 25). The results reported in this paper show that an enhancement of growth of a myxovirus can also be achieved by proflavine. This enhancement of growth was obtained only by employing a low MOI and could only be detected two days after infection of the tissue culture at a time when a second generation of virus growth must have taken place. *Shigeta et al.* (1968) have shown that a major part of the spread of RS virus in HEp 2 cell cultures can take place by direct transfer from cell to cell. It remains a possibility that proflavine promotes the cell to cell transfer of RS virus.

The results of AO staining of RS virus infected cells described in this paper confirm the results obtained by *Kisch et al.* (1962) that no inclusion bodies and no change in intensity of RNA or DNA staining could be seen in RS virus infected cells stained by this method. After incubation of RS virus infected HEp 2 monolayers with a low concentration of actinomycin D for 2–3 days AO staining nevertheless revealed that RNA accumulated in syncytia and in a number of single cells in these cultures at the same time as parallel noninfected cultures lost most of their stainable RNA. This technique confirms that the multiplication of RS virus is independent of host cell directed RNA synthesis and takes place in the cytoplasm.

SUMMARY

The influence of proflavine and of actinomycin D on the multiplication of RS virus (and adenovirus) in HEp 2 cells has been studied.

Proflavine was able to promote the growth of RS virus when a low MOI was employed and this compound was added after an absorption period of three hours. This increased virus yield induced by proflavine was evident two days after infection of the HEp 2 cells.

A concentration of actinomycin D which inhibited the growth of adeno (type 3) virus did not influence the growth of RS virus when

this compound was added either before after or together with this virus

AO staining of RS virus infected HEp 2 cells incubated with actinomycin D showed that RNA accumulated in the cytoplasm of virus infected cells (syncytia) at a time when noninfected cells had lost most of their RNA

The differences in effect of proflavine and actinomycin D are discussed

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BRIEF REPORT

ANTIBODIES AGAINST MYCOPLASMA PNEUMONIAE IN BRONCHIAL SECRETIONS

By *Gunnel Biberfeld* and *Goran Sterner*

Antibodies present in respiratory secretions are considered to be important for resistance to respiratory infections (4) *Antibodies of the IgA class usually predominate* in respiratory secretions (4)

Mycoplasma pneumoniae is an important respiratory pathogen in man (3) *M pneumoniae* often persists in the respiratory tract for several weeks or months Previous attempts to demonstrate *M pneumoniae* antibodies in nasal secretions were unsuccessful (5) The present preliminary communication describes the demonstration of antibodies in sputa from cases of *M pneumoniae* infection

Sputum specimens from 16 cases of respiratory disease associated with *M pneumoniae* infection were examined Thirteen of these cases showed a fourfold or greater rise of complement fixing (CF) antibodies in serum during the course of infection and the remaining 3 cases from which only convalescent phase sera were available had *high titres* ($\geqq$ *1/256) of CF antibodies to M pneumoniae In addi*tion sputa from 6 cases of respiratory infection lacking CF antibodies to *M pneumoniae* in serum were tested Sputum specimens were diluted ¼ in phosphate buffered saline homogenized and centrifuged at 2000 r p m for 30 minutes The supernatant was used for antibody and immunoglobulin determinations Antibodies were measured by the indirect immunofluorescence (IMF) technique and the complement fixation (CF) test Colonies of *M pneumoniae* transferred to glass slides were used as antigen in the IMF test (1) The following fluoresceinconjugated antisera were used a sheep antihuman gammaglobulin serum reactive with IgA IgG and IgM and monospecific rabbit antisera to IgA IgG and IgM respectively Immunoglobulin concentrations were determined by the single radial diffusion *method* (2)

Fourteen out of 16 cases of *M pneumoniae* infection had demonstrable IMF antibodies in sputum In some cases a fourfold or greater increase of sputum antibodies was demonstrated during the course of infection Sputa from 9 cases were examined in the IMF test with monospecific antisera to *IgA and IgG* All these cases were found to have both IgA and IgG antibodies to *M pneumoniae* in sputum In addition sputum antibodies of the IgM class were demonstrated in 3 out of 7 cases Serum antibodies were of all three immunoglobulin classes Antibody titres and immunoglobulin levels in some representative cases are given in Table I *The* IgA levels would probably have been about 3 times higher than the values recorded in the table if an 11S IgA preparation had been used as standard instead *of a 7S IgA (4)*

Eight out of 9 cases of *M pneumoniae* infection examined for sputum CF antibodies were found to have such antibodies These antibodies could only be IgG or IgM since IgA antibodies do not fix complement (4)

M pneumoniae was isolated from throat or sputum specimens of 6 cases which at the same time had IMF antibodies to *M pneumoniae* in sputum

Six seronegative control cases with respiratory infections lacked IMF and CF antibodies against *M pneumoniae* in sputum

Antibodies have been demonstrated in respiratory secretions following infection or immunization with several different respiratory pathogens (4) The occur

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TABLE 1

IMF and CF Antibody Titres in Sputa and Sera and Immunoglobulin (Ig) Levels in Sputa of Cases with M pneumoniae Infection The Titres are expressed as the Reciprocal of the Dilution

Case No	Days after onset of respiratory illness	Specimen	Antibody titres IMF IgA	IgG	IgM	CF	Ig levels mg/100 ml IgA	IgG	IgM
1	20	sputum	8	16	nd§	8	13	36	nd
	30	sputum	8	16	4	8	20	70	trace
	20	serum	128	512	nd	512			
	30	serum	128	512	1024	256			
2	5	sputum	< 2	< 2	8	4	28	108	14
	12	sputum	16	8	32	8	18	21	13
	5	serum	8	16	128	64			
	12	serum	64	256	1024	512			
3	9	sputum	16	16	nd	nd	26	2[illegible]	nd
	23	sputum	8	8	< 2	nd	9	6	nd
	9	serum	64	128	32	32			
	23	serum	512	1024	512	256			
4	10	sputum	4	2	< 2	nd	62	11[illegible]	7
	53	sputum	4	4	nd	nd	33	28	nd
	10	serum	8	64	16	16			
	25	serum	128	256	64	64			
	53	serum	64	128	32	32			
5	23	sputum	4	2	< 2	8	34	18	—
	23	serum	64	256	16	256			
6	2[illegible]	sputum	16	8	8	32	2[illegible]	26	16
	25	serum	32	256	512	512			

A 7S IgA was used as standard
§ nd = not done

rence of antibodies in secretions from cases of *M pneumoniae* infection was therefore to be expected However sputum antibodies to *M pneumoniae* were of both IgA and IgG class and in some cases also of IgM class whereas viral antibodies in respiratory secretions have been shown to be associated primarily with IgA (4) It is also noteworthy that *M pneumoniae* organisms persisted in the respiratory tract in the presence of specific secretion antibodies

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Acta path microbiol scand 76 648 650 1969

BRIEF REPORT

ATYPICAL ADENOVIRUS TYPE 1 STRAINS ISOLATED FROM PATIENTS WITH LARYNGITIS

By C Stuart Malmberg and E Norrby

In the course of a study on the aetiology of subglottical laryngitis (pseudocroup) in children (6) three adenovirus strains were isolated which could not be typed by the neutralization technique routinely employed for type identification of adenoviruses During the same period one similar adenovirus strain was isolated from an adult who among other symptoms suffered from an acute laryngitis with severe hoarseness The results of virological characterization showed that these four adenovirus strains belonged to Rosen s subgroup III (4) However a n n parallelism between neutralization reactions and haemagglutination inhibition re actions was demonstrated

All four strains were isolated from throat swabs two of these (4905 and 4930) in a human fibroblast strain and the other two (4960 and 4978) in HeLa cells The neutralization technique employed was devised by *Kjellen et al* (?) and its application to the typing of isolated virus strains was described by *Sterner et al* (5) This technique is based upon the capacity of a serum to delay the appearance of adenovirus cytopathic effects (CPE) in cell cultures which is taken as a measure of the neutralizing activity of the serum Virus strains were used in a concentration known to give an extensive cytopathic degeneration within one to two days after inoculation Typing sera were used at a dilution which caused a homotypic delay of appearance of this degeneration of at lea t 10 days A minimum delay of 7 days was required for type identification of an unknown strain Typing sera were produced in rabbits which were injected with antigens prepared from prot type strains The haemagglutination inhibition (HI) tests were performed accord ing to *Rosen* (4) Recently washed rat cells the agglutinability of which was controlled in tests with a type 6 stock material were employed Complement fixation tests were carried out according to *Svedmyr et al* (7) Data concerning the four patients are presented in Table 1 The adult patient besides his severe laryngitis displayed a swelling of both parotid glands and signs of liver disease Liver biopsy revealed degenerative changes Liver function tests returned to normal values in about a month Serum specimens for vir logical examination were col lected 12 and 20 days respectively after the onset of the disease There was no titre rise of complement fixing antibodies to aden virus mumps or cytomegalovirus Both sera however neutralized the isolated adenovirus strain for at least 7 days —Serum specimens were not available from the three children

The virus strains were all isolated from specimens collected within the same month (April 66) Both the HeLa cell line and the fibroblast strain were u ed for virus studies before and after this period without appearance of similar strains Reisolation was not attempted until 17 months after the collection of the pe imens The two strains isolated in HeLa cells were then recovered again as well as the parainfluenza type 3 strain

All four strains were identified as adenovirus strains by complement fixation tests against a pool of human convalescent sera as well as against a guinea pig immune serum However the strains were not neutralized by any of the rabbit immune sera to adenovirus types 1–7 and 9–17 prepared at the Virus Department of Karolinska sjukhuset On the other hand all four isolates were found t behave like type 1 in HI tests When these data were btained the strains were reexamined by neutralization tests using both the type 1 serum prepared at the Virus Depart

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ment of Karolinska sjukhuset (Ad 1 KS) and a type 1 serum prepared at the Department of Virology of Karolinska Institutet (Ad 1 KI) as well as a rabbit immune serum prepared to one of the four isolated strains (4930). The results of these tests are shown in Table 2. As can be seen the behaviour of the different isolates was clearly distinguishable from that of prototype 1.

The finding that one of the specimens contained *type 3 parainfluenza virus* in addition to adenovirus led to examination for haemadsorption and haemagglutination of guinea pig erythrocytes of several different passages of the other three strains. These tests were invariably negative.

TABLE 1

Clinical Data on the Patients from whom Atypical Adenovirus Type 1 Strains were Isolated

Strain No	Patient aged	Diagnosis	Anamnestic data
4905	59 y	Acute laryngitis, suspect parotitis, fatty degeneration of the liver	One year earlier similar symptoms but not hospitalized
4930	2 y 8 mo	Subglottical laryngitis (pseudocroup)	Recently parotitis. Hospitalized once before because of pseudocroup
4960	2 y 2 mo	Subglottical laryngitis (pseudocroup)	
4978 (+ para influenza virus type 3)	1 y 2 mo	Subglottical laryngitis (pseudocroup)	Hospitalized once before because of pseudocroup

TABLE 2

Serologic Tests with the Atypical Adenovirus Type 1 Strains and with the Prototype Strain

Strain No	Specimen collected	Reisolated Sept 67	Neutralization test No of days of delay of adenovirus CPE by serum: Ad 1 KS diluted 1:10	Ad 1 KI diluted 1:5	Anti 4930 diluted 1:10	HI test Reciprocal of highest dilution of serum causing inhibition: Ad 1 KI	Anti 4930
4905	April 66	No	3	3	8	640	320
4930	April 66	No	4	3	10	320	320
4960	April 66	Yes	3	4	10	320	320
4978	April 66	Yes (both strains)	§		§	320	320
						320	320
Adenovirus type 1	prototype		12	12	7	640	320

Parainfluenzavirus type 3 isolated from the same specimen.

§ The simultaneous presence of parainfluenzavirus precluded the possibility to read adenovirus CPE.

The most probable explanation that can be derived from the present laboratory data is that at least the three strains 4905 4930 and 4960 share some property distinguishing them from the type 1 prototype in the neutralization test but not in the HI test Since these two tests are based on type specific antibodies reacting with hexons and fibres respectively (cf 3) it seems likely that differences regarding properties of the former structural component are responsible for the present findings The same type of results have previously been obtained in studies of some members of Rosen s subgroups I and II but not of subgroup III The only variant of a subgroup III member which has been described was an isolate of type 5 This isolate displayed a markedly increased cross reactivity with type 1 in neutralization and HI tests (1) None of the strains described above displayed an immunological relationship to prototype 5

Parainfluenza virus type 3 is not an unexpected finding in a child with subglottical laryngitis There were 9 more patients out of those investigated during the same period from whom this virus was isolated (6) In some cases the parainfluenza type 3 infection was verified serologically as well There is no serological evidence to support a hypothesis that the atypical adenovirus type 1 strains isolated were the cause of the disease of the patients One can only speculate on the possibility of an unrecognized agent activating a latent adenovirus infection and taking part in the pathogenesis of the laryngitis

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TRANSACTIONS OF THE SWEDISH PATHOLOGICAL SOCIETY

Meeting April 26, 1969

Thorell B ENZYME KINETICS IN THE LIVING CELL

Pyridine nucleotide (DPN or NAD) participates as coenzyme in a variety of oxidative reactions which supply energy to the cell These reactions involve reduction oxidation transitions of the coenzyme (NAD NADH) The reduced state exhibit a characteristic blue fluorescence which intensity can be measured with a microfluorimeter within localized parts (nucleus mitochondria and extramitochondrial space) of the single living cell

A beam splitter microfluorimeter is used for the kinetic assay of NAD NADH Association with various enzymatic reactions in the different cellular compartments is achieved by the microinjection of selected glycolytic or Krebs cycle substrates For instance 1 mM of glucose 6 phosphate given to a 1 $\mu\mu$ 1 cell causes a fluorescence pulse" in the extramitochondrial space with a peak level of 200–400 per cent over initial value and a half life (period) of 10 seconds The pulse represents the summation of mainly two enzymatic reactions Rising part = NAD reduction at glyceraldehyde phosphate dehydrogenase and decending part = reoxidation at lactic dehydrogenase

The microfluorimetric microinjection technique has allowed the study of interaction or competition between the different metabolic compartments of the cell metab lic comparison between different types of cells such as normal and tumour cells modifications of intracellular metabolic patterns by hormones drugs X irradiation etc

Heglen (Oslo) ENZYME HISTOCHEMICAL FINDINGS IN HARD TISSUES AND SOME METHODOLOGICAL RESULTS

Willighagen R G J (Leiden) ENZYME HISTOCHEMISTRY OF HUMAN TUMOURS

Discussion

Hägerstrand I & Nordén J G A biopsy from a liver tumour was presented A diagnosis of a hepato-cellular carcinoma was made with support from the staining reactions for acid phosphatase and gammaglutamyltranspeptidase by their capacity to visualize bile canalicular structures of the tumour

Jonsen K F Hagerstrand I Nordén J G & Pihl B ENZYME HISTOCHEMISTRY OF THE LIVER IN TUMOUR DISEASES

The serum activity of alkaline phosphatase and gammaglutamyl transpeptidase increases in cases of tumour growth involving the liver Biopsies from 25 patients with tumour diseases of the liver were examined by staining reactions for these

two enzymes The biopsies included tumour growth affecting surrounding hepatic tissue and in some cases liver tissue free from tumour growth An increased enzyme activity was demonstrated in hepatic tissue mainly in the periphery of tumour growth in canaliculi for gammaglutamyl transpeptidase along sinusoids for alkaline phosphatase It was concluded that an increased enzyme activity from hepatocytes and a pathological passage of enzymes produced in biliary tracts to the blood were the two possibilities to be regarded a an explanation of the serum increase in enzyme activity

Aronsen K F Hagerstrand I Norden J G & Pihl B ENZYME HISTOCHEMISTRY IN BILE STASIS

In extrahepatic biliary stasis the reaction for alkaline phosphatases in the liver was increased along the sinusoids *in man dog and rat and further* in the d g markedly so in the bile canaliculi already after one day ATP ase was increased in the three species sinusoidally but was often diminished in the canaliculi L leucyl *aminopeptidase has a weak reaction in the rat* in man *a moderate* sinusoidal *increase and a marked reaction in dilated canaliculi* with visualization of diverticula The acid phosphatase reaction was in all three species increased in peribiliary lysosomes Dogs with one month of biliary stasis showed acid phosphatase positive round bodies in the intermediary zone somewhat smaller than *hepatocytes In human livers in association with clumps of bile pigment* in the centrolobular zone there were irregular homogenously acid phosphatase positive bodies bigger than hepatocytes—Enzyme reactions with complete canalicular hexagons exclude the old concept of hepatocellular trabeculae and demonstrate the liver cell plates

Ljungberg O HISTOCHEMICAL STUDIES ON MEDULLARY THYROID CARCINOMA

Medullary thyroid carcinoma from two cousins who had also unilateral phaeochromocytomas were studied with the following histochemical methods for demonstration of certain mono amines the argentaffin chromaffin and iodate reaction (according to *Hillarp Hokfelt*) fluorescence reaction (according to *Falck Hillarp*) and the diazo and ninhydrin reaction In both tumours argentaffin cells with long cytoplasmatic extensions were demonstrated often localized in close relationship to blood vessels Positive chromaffin as well as positive iodate *reaction were seen in unevenly* distributed cell The fluorescence method showed cells with a green intracytoplasmatic fluorescence characteristic of primary catecholamines and certain closely related substances The diazo and ninhydrin methods for demonstration of 5 hydroxy tryptamine were negative The study thus *demonstrated the existence of mono amine containing cells* in the thyroid tumours and lends support to the hypothesis that medullary thyroid carcinoma is a neuroepithelial tumour or at least contains a neuro epithelial cell component

Ericsson J & Arborgh B THOROTRAST INDUCED SYNTHESIS OF LYSOSOMAL ENZYMES IN RAT LIVER

Current evidence indicates that endocytosis vesicles move toward and fuse with lysosomes This concept is mainly based on fine structural studies of the uptake of electron dense or radioactively labelled macromolecules

In order to study the morphological and biochemical effects of induced endo-

citosis on lysosomes and lysosomal enzymes experiments were performed in which thorium dioxide (thorotrast) particles were injected intravenou ly into rats, and quantitative biochemical measur ments of lysosomal enzymes were correlated with the f ne structural findings Electron microscopy showed that thorotrast particles were rapidly endocytosed by the Kupffer cells which became loaded with par ticle containing vacuoles appr ximately 30 minutes after the injection Many of the thorotrast filled vacuoles showed presence of acid phosphatase At later inter als (1 ? 4 1? ?4 and 7 < ?4 hours after the injection) the number and size of thorotrast vacuoles was approximately the same as at 30 minutes however all the vacuole appeared to contain lysosomal enzyme (aci l phosphatase) Hepatic parenchymal cell lysosomes took up thorotrast particles at a slow rate and did not show appreciable increase in size or number

Biochemically total activity of two lysosomal enzymes (cathepsin D and aryl sulphatase) in homogenates of the liver was slightly *diminished 2 and 6 hours* after the injection and moderately (?0-2? per cent) increased after ?4 hour Seven d ys after thorotrast induction of endocytosis in the liver total *enzyme* activity (as calculated per mg of protein) was more than doubled The *thoro* trast induced increase in lysosomal enzyme activity could be blocked by actino *mycin D* Following cessation of actinomycin D effects a rise in lysosomal *en* zymes was recorded

The finding indicate (*1*) that endocytosis vacuoles and lysosomes *interact and* mix their contents whereby the absorbed materials are brought *in contact* with lysosomal enzyme (?) that the in rease in lysosomal enzymes is due to (gene activated) indu tion of synthesis *and (3)* that the increase in size and number of secon lary lysosom s (rather than *the endocytosis* process *per se*) triggers the induction of synthesis

Vejlens L CHONDROITIN SULPHATE PATTERN IN FEMORAL CORTEX AND MEDULLA IN OSTEOPETROSIS

Bergstrand A FUNCTIONAL AND MORPHOLOGICAL RELATIONS BETWEEN RIBOSOMES AND MICROSOME MEMBRANES

Eriksson L TURNOVER OF MICROSOMAL MEMBRANE COMPONENTS

Dallner G THE EARLY EFFECT OF CARCINOGENS ON THE ENDOPLASMIC MEMBRANES OF THE LIVER

Glaumann H SYNTHESIS AND TRANSPORT OF ALBUMIN

The liver microsomal fra tion is made up of a number of vesicles which derive from the rough n l smooth urfaced endoplasmic reticulum This organelle is the maj r site of serum protein s nthesis *The synthesis* and transp rt of albumin in the liver cell were studied after inject on of ^{14}C leucine into v m senters a su r The microsomes were subfractionated on a discontinous ioncontaining sucr se gradient system int rough smooth I and smooth II membranes. Th subfractionated microsomes were sonicated and the albumin i olated on disc electrophores The albumin cont nt on a membrane protein basis of rough micro

somes is 0 1 that of the smooth I 0 13 and that of the smooth II fraction 0 17 After 2 min of incorporation the albumin of rough membranes shows about 7 times the activity of the smooth counterparts The curve for rough membranes reaches a maximum at 6 min followed by a rapid decrease which is accompanied by an increase of two smooth fractions with maxima at about 20 min 5 min later radioactive albumin appears in the blood *There are no differences in the* time curves of the smooth I and II microsomes which both participate in the process Cycloheximide (0 05 mg/100 g) inhibits the protein synthesis almost completely a few minutes after venous administration Injection of cycloheximide two min after administration of ^{14}C leucine shifts the incorporation maximum of rough microsomes from 6 to 3 min and that of the smooth I and II microsomes from 20 min to 14 min These results demonstrate that the rough part of *the endoplasmic reticulum is the site of albumin synthesis followed by transport* to the smooth counterparts where the protein remains for about 10 min before being liberated into the blood

Jakobsson S DOES THE KIDNEY PARTICIPATE IN THE DETOXICATION OF SLEEPING DRUGS?

Dahl I & S ogi S LYMPH NODE BIOPSIES IN A COUNTRYSIDE HOSPITAL

Berge Th LYMPH NODE METASTASES IN FOSSAE SUPRACLAVICULARES AND MEDIASTINUM

The frequencies of metastasis to the supraclavicular and mediastinal lymph nodes were studied in an autopsy series consisting of approximately 4 000 carcinomas Mediastinal lymph node metastases were significantly ($p < 0\,001$) more common than metastases to the left supraclavicular fossa Metastases to the left supraclavicular fossa were more common than those to the right fossa ($p < 0\,001$) The greatest number of metastases to all the three locations were produced by carcinomas of lung breast and stomach respectively By means of a frequency list and the histological picture the pathologist should be able to guide the clinician in his search for the site of the primary tumour

Zettergren I LIPO FUSCINOSIS IN ABDOMINAL LYMPH NODES

Brandt P & Rausing A LYMPHOPROLIFERATIVE DISEASE WITH AMYLOIDOSIS

Amyloidosis is a well known complication of myelomatosis It may also be seen with lymphoproliferative disease with electrophoretically homogenous immunoglobulin—spikes Three cases were described one being a 69 year old woman with a lymphatic leukaemia like disease with a macroglobulin spike who developed generalized amyloidosis The second case was a 90 year old man with prostatic bleeding and F S R 150 mm/hr who at autopsy showed lymphoplasmacytoid infiltrates with amyloid material in the retroperitoneal tissue No electrophoresis performed Case no three was a 50 year old woman with classical Waldenstroms macroglobulinaemia since the age of 35 At autopsy polymorphous lymphoreticular infiltrates in the pelvis with localized amyloidosis

Rausing A PROGRESSIVE MULTIFOCAL LEUCOENCEPHALOPATHY—POLYOMA VIRUS

Progressive multifocal leuco encephalopathy is a demyelinating disease usually seen as a complication in malignant bone marrow or lymph node disease Polyoma virion like structures in the diseased glial cells have been seen in the electron microscope A case is described confirming this finding for the first time in Scandinavia

Brehmer Andersson E Månsson T Willbeck B & Grubb R AQUARIUM ACQUIRED INFECTION OF MYCOBACTERIUM MARINUM

Knutson F Lundin I M & Norrby K ON THE VIABILITY OF ENZYMATICALLY DISSOCIATED TUMOUR CELLS IN SUSPENSIONS DURING SHORT TIME STORAGE

The aim of this work was to study the effect of various media and temperatures on the viability of enzymatically produced mouse sarcoma suspensions during short time storage *in vitro* The viability after 1 3 4 and 21 hours at 0 22 and 37 C was measured as the total number of viable cells using cell counts and the trypan blue exclusion test In some experiments autoradiographic determinations of the incorporation of tritiated thymidine were also performed. The *in vitro* test model used seems to be of relevance in most tumour cell and tissue transplantation procedures

Suspensions stored at 37 C showed a reduced viability Storage at 0 C constantly showed the highest viability but marked differences against suspensions stored at 22 C did not always appear until after 3 hours

Storage of suspensions in synthetic medium 199 always presented reduced viability as compared with protein containing media Suspensions in pure 199 stored at 0 C also showed a further loss of about 10 per cent viable cells after warming for 3 minutes at 37 C a loss that did not appear in suspensions in protein containing media

The protein containing media tested contained 199 with 20 per cent foetal bovine serum syngeneic mouse serum and syngeneic ascitic fluid respectively No definite differences between these three media were observed

Preliminary results indicate that the growth and distribution of experimental metastases *in vivo* is influenced by the storage conditions of the test suspensions

Hagmar B & Boeryd B ACTION OF HEPARIN AND COUMARIN ON THE DISTRIBUTION OF EXPERIMENTAL METASTASES

In allogeneic systems anticoagulants have previously been reported to reduce metastasis production from intravenously injected tumour cells On this basis thromboses found histologically around tumour cells in vessels have been considered important or necessary for metastasis formation

Now the effects of heparin and a coumarin anticoagulant phenprocoumon were tested on intravenously induced metastases to two syngeneic tumour host systems (CGI SS in CBA mice and melanoma B16 in C57 Bl/6J mice

Heparin reduced pulmonary metastasis formation in both systems obviously by increasing the transpulmonary passage of tumour cells This effect noted as an

increase of extrapulmonary metastases became evident in the case of melanoma B16 only after the cell dose was raised and the survival time prolonged

Phenprocoumon increased the total outcome of metastases to both systems In the case of melanoma B16 this occurred in the lungs in the case of MCGI SS it occurred extrapulmonally

The results indicate that thrombus formation contrary to earlier concepts rather impairs the development of metastases

Heparin promotes the dissemination of tumour cells probably by a mechanism other than impaired blood coagulation A binding to the tumour cell membranes seems at present to be the most likely explanation

Lundberg S & Berge Th MICROSCOPIC CARCINOMA OF THE PROSTATE

In a consecutive autopsy series of 292 men aged 40 years and more clinically unsuspected microscopic carcinoma was found in 116 (39 6 per cent) After 80 years of age the frequency was 44 per cent Early carcinoma was found in the periphery of the prostate without preference to any lobe The number of sections with carcinoma increased according to age and so did growth in perineural spaces

Mikulowski P PURE SARCOMA OF THE BREAST

A retrospective study of pure mammary sarcomas diagnosed at the University Department of Pathology in Malmo between 1956 and 1968 is presented Among about 1 200 mammary malignant tumours 5 cases of sarcoma without epithelial proliferation were found Clinical and pathological data of 2 malignant lymphomas, 1 osteogenic sarcoma 1 angiosarcoma and 1 alveolar rhabdomyosarcoma are discussed

The biological properties of breast sarcomas are similar to those of sarcomas elsewhere in the body and histological classification seems to be most important for prognosis and adequate treatment Radical mastectomy is indicated only in cases of malignant lymphomas and rhabdomyosarcomas

Lowhagen O Enerback L & Säve Söderberg J GENERALIZED MASTOCYTOSIS

Lindstrom C G DESQUAMATIVE INTERSTITIAL PNEUMONIA (DIP)

Two cases of DIP first described by *Liebow et al* in 1965 have recently been diagnosed in Malmo on open lung biopsies They seem to be the first cases of DIP reported from Scandinavia

The patients are males 24 and 46 years old respectively with increasing dyspnoe as the main symptom The younger had also clubbing and the older dry cough and an episode of bilateral spontaneous pneumothorax X ray showed a diffuse ground glass like density in the first patient most pronounced on the lung bases

Physiologically there was respiratory insufficiency of restrictive type with signs of low diffusing capacity Histologically the alveoli and distal air spaces were filled by large eosinophilic PAS positive granular pneumocytes with scattered multinucleated giant cells of the same type and with some mitoses Thickening of the alveolar walls was absent or only slight in the first patient but was present in the other There was scattered interstitial round collections of lymphocytes

Steroid therapy has had good effect in both patients